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# Factors influencing pneumonia and *Mycoplasma hyopneumoniae* infections in pigs

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Certain things catch your eye, but pursue only those that capture the heart. – Ancient Indian Proverb

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| | |
|---|---|
| #: | number |
| %: | percentage |
| 15-ADON: | 15-acetyldeoxynivalenol |
| 3-ADON: | 3-acetyldeoxynivalenol |
| *A. pleuropneumoniae:* | *Actinobacillus pleuropneumoniae* |
| Ab: | antibodies |
| ADG: | average daily gain |
| AFLP: | amplified Fragment Length Polymorphism |
| AMCRA: | Antimicrobial Consumption and Resistance in Animals |
| ANOVA: | one way analysis of variance |
| AR: | atrophic rhinitis |
| av.: | average |
| *B. bronchiseptica:* | *Bordetella bronchiseptica* |
| BALF: | bronchoalveolar lavage fluid |
| BCFP: | bacterial colony forming particles |
| BHI: | brain heart infusion |
| BW: | body weight |
| CCU/ml | colour changing units per ml |
| $CH_4$: | methane |
| CI: | confidence interval |
| $CO_2$: | carbon dioxide |
| CT: | computed tomography |
| d: | days |
| D: | day of the study |
| DNT: | dermonecrotic |
| DON: | deoxynivalenol |
| E: | experimental study |
| EP: | enzootic pneumonia |
| *F. graminearum:* | *Fusarium graminearum* |
| F: | field study |
| FB1: | fumonisin B1 |
| FB2: | fumonisin B2 |
| FB3: | fumonisin B3 |
| *H. parasuis:* | *Haemophilus parasuis* |
| $H_2S$: | hydrogen sulphide |
| HPLC-$H_2O$: | high performance liquid chromatography filtered water |

| | |
|---|---|
| ICEF: | Integrative Conjugal Element of Mycoplasma fermentans |
| IF: | immunofluorescense |
| LC-MS/MS: | liquid chromatography-tandem mass spectrometry |
| *M. flocculare:* | *Mycoplasma flocculare* |
| *M. hyopneumoniae:* | *Mycoplasma hyopneumoniae* |
| MIN: | liquid mineral |
| MLL: | macroscopic lung lesions |
| MLST: | Multiple-Locus Sequence Typing |
| MLVA: | Multiple-Locus of variable number of tandem repeats |
| MØ: | macrophage |
| MST: | minimal spanning tree |
| N.A.: | not applicable |
| NCG: | negative control group |
| $NH_3$: | ammonia |
| nPCR: | nested Polymerase Chain Reaction |
| NPTr: | newborn pig trachea epithelial cell line |
| OD: | optical density |
| OR: | odds ratio |
| *P. multocida:* | *Pasteurella multocida* |
| P: | p-value |
| PAM: | porcine alveolar macrophages |
| PBS: | phosphate buffered saline |
| PCM: | Pig Cough Monitor |
| PCR-RFLP: | PCR-Random fragment length polymorphism |
| PCV2: | Porcine Circovirus type 2 |
| PCVAD: | porcine circovirus associated disease |
| PFGE: | Pulsed-Field Gel Electrophoresis |
| PI: | post inoculation |
| PM: | particulate matter |
| PPLO: | phenol red, pleuropneumonia like organism enrichment |
| PPRSv: | Porcine Reproductive and Respiratory Syndrome virus |
| PRDC: | Porcine Respiratory Disease Complex |
| qPCR: | quantitative real-time Polymerase Chain Reaction |
| RAPD: | Random Amplified Polymorphic DNA |
| RDS: | respiratory disease score |
| *S. suis:* | *Streptococcus suis* |
| SE: | standard error |
| SIV: | swine influenza virus |

| | |
|---|---|
| UK: | United Kingdom |
| US: | United States |
| VNTARs: | variable number of amino acid repeats |
| VNTR: | variable number of tandem repeat |
| wk: | weeks old |

# CHAPTER 1: GENERAL INTRODUCTION

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## *Review of the literature*

With the intensification of swine production, respiratory diseases have become more responsible for economic losses. One of these economic drains is caused by enzootic pneumonia (EP): a chronic respiratory disease with a worldwide distribution caused primarily by *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) and secondary bacterial pathogens (Kobisch and Friis, 1996; Kobish et al., 1993; Maes et al., 2008; Maes et al., 2017; Ross, 1999). *Mycoplasma hyopneumoniae* acts as a triggering factor for infection with these secondary bacterial pathogens (Maes et al., 2008). *Mycoplasma hyopneumoniae* is considered as a major pathogen in the Porcine Respiratory Disease Complex (PRDC) as well, next to other respiratory bacterial, parasitic and viral pathogens, management and environmental conditions (Opriessnig et al., 2011). Economic losses are mainly due to treatment and vaccination costs, decreased performance, such as reduced growth and increased feed conversion ratio, increased time to market and higher mortality rates, mostly caused by infection with secondary agents (Maes et al., 2008; Maes et al., 2017).

*Mycoplasma hyopneumoniae* is a member of the Mollicutes and is not able to survive long outside the host, which is a consequence of the fact that the organism lacks a cellular wall. Nevertheless, its survival time can be extended for up to 31 days in water at 2-7°C (Goodwin, 1972).

As eradication of *M. hyopneumoniae* is difficult and expensive (culling of pigs, costs for antimicrobial therapy) and reinfection of a *M. hyopneumoniae*-free herd is common, control of the disease is probably the best strategy, especially in pig dense areas such as Western-Europe (Maes et al., 2017). Optimizing housing and management practices, implementing vaccination strategies, not only to control *M. hyopneumoniae*, but also other pathogens considered important in PRDC, should be advised.

This introduction aims to summarize the current knowledge on the following aspects of *M. hyopneumoniae* infections: characteristics of the pathogen, pathogenesis of the infection, parameters to assess the severity of *M. hyopneumoniae*-infection and factors influencing the outcome and severity of the disease.

## *1.1. Characteristics of Mycoplasma hyopneumoniae*

*Mycoplasma hyopneumoniae* belongs to the division Firmicutes, class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae, genus *Mycoplasma* (Peters et al., 2008). It has no cellular wall and a small genome (893-920 kilobase pairs) (Dybvig and Voelker, 1996). As a result, a limited number of metabolic pathways are encoded by a small number of genes, resulting in *Mycoplasma* species needing to obtain essential metabolites from the host or the environment (Razin et al., 1998; Thacker and Minion, 2012).

In the 1930's Köbe described a chronic and prevalent EP in Germany. This disease was distinct from Shope's swine influenza and the name 'Ferkelgrippe' was given to the disease (Köbe, 1936). After the report of Köbe in 1933, several researchers reported similar EPs in several countries (Fulton et al., 1953; Hjärre et al., 1952; Plowright, 1953; Rislakki, 1953). The aetiology was not elucidated at that time, due to the historical misleading preconception that the causative agent of these EPs reported in pigs was a virus, the difficulty in obtaining pigs

for experimental inoculation that were free from respiratory agents and because the inoculation material often was infected with *Mycoplasma hyorhinis (M. hyorhinis*) which grows faster than *M. hyopneumoniae* and is a common secondary bacterial agent in EP (Takatori, 1970). Thirty years after the discovery of the disease EP, Maré and Switzer (1965), in the United States (US), and Goodwin et al. (1965), in the United Kingdom (UK), were able to isolate the organism on solid medium. The organisms isolated in the US and UK were called *M. hypopneumoniae* and *M. suipneumoniae,* respectively (Goodwin et al., 1965; Maré and Switzer, 1965). Afterwards, it was concluded that both mycoplasmas were serologically indistinguishable, and the name *M. hyopneumoniae* remained (Goodwin et al., 1967; Hodges et al., 1969; Rose et al., 1979; Takatori, 1970). Before the aetiology of EP was known, the J-strain was isolated in 1963 from a field outbreak in sows experiencing a mild course of EP (Goodwin et al., 1967; Goodwin and Whittlestone, 1963). This strain is still considered to be the reference strain of *M. hyopneumoniae*.

*Mycoplasma hyopneumoniae* grows very slow *in vitro* and requires a growth medium composed of Hank's balanced salt solution, fresh acetic yeast extract from *Saccharomyces cerevisiae*, ampicillin, bacitracin, phenol red, pleuropneumonia like organism enrichment (PPLO) broth base, brain heart infusion (BHI) broth, porcine and horse serum (Friis medium) (Friis, 1975). The *M. hyopneumoniae* cultures can easily be overgrown by other bacteria of the respiratory tract, typically *M. hyorhinis* and by any environmental organism (Friis, 1975; Thacker and Minion, 2012). Minor fluctuations in the composition of the medium, may influence growth of *M. hyopneumoniae* (Calus et al., 2010). According to Madsen et al. (2007), especially the swine serum component is highly variable in supporting growth of recent *M. hyopneumoniae* isolates.

## *1.2. Pathogenesis of Mycoplasma hyopneumoniae infections*

The pathogenesis of *M. hyopneumoniae* infections is complex and not yet fully elucidated. Especially the role of specific virulence factors needs more attention (Simionatto et al., 2013; Thacker and Minion, 2012). To date, only domestic pigs and wild boar are known hosts for infection with *M. hyopneumoniae* (Maes et al., 2017). *Mycoplasma hyopneumoniae* infections are characterized by a long lasting persistence in the airway tract, a prolonged inflammatory reaction, modulation and suppression of the innate and adaptive immune system, and interaction with other (secondary) respiratory agents (Del Pozo Sacristán, 2014; Thacker and Minion, 2012). First, *M. hyopneumoniae* needs to establish istself at the appropriate site of infection after inhalation of *M. hyopneumoniae*-infected respiratory aerosols, expelled by infected pigs. A mutualistic relationship with the host is obtained when the pig is merely colonised, not infected (Dani, 2014; Desrosiers, 2001; Maes et al., 2017; Simionatto et al., 2013; Thacker and Minion, 2012). In certain circumstances, infection is established and disease signs are observed. It is unclear why in certain pigs, *M. hyopneumoniae* might remain in the respiratory tract without causing clinical signs, or which factors are involved in progressing from colonisation to infection (Dani, 2014). Colonised pigs might be important from point of view of disease transmission in case of *M. hyopneumoniae*.

The bacterium is situated at the apex of the cilia, is adhered to the microvilli or is situated in the interciliary space. A minority of mycoplasmas reach the smaller bronchioles and alveoli but they do not invade the airway epithelium (Blanchard et al., 1992; Jaques et al., 1992; Maes et al., 2017). Debey and Ross (1994) demonstrated that attachment of *M. hyopneumoniae* to ciliated epithelium was necessary to induce ciliostasis and loss of cilia. The exact mechanism of adhesion to the cilia has not yet been fully elucidated. The first identified adhesin was P97,

but several other adhesins belonging to the P97/P102 families have been identified (Simionatto et al., 2013). An overview of these adhesins is provided by Simoniatto et al. (2013). The adhesin P159 is unrelated to the P97/P102 paralogues (Burnett et al., 2006). All members of the P97/P102 family and P159 are post-translationally cleaved. These cleavage products on the surface of *M. hyopneumoniae*, including adhesins, lipoproteins and also cytosolic proteins 'moonlighting' at the cell surface represent an important mechanism for creating cell surface protein diversity in genome-reduced bacteria such as *M. hyopneumoniae* (Maes et al., 2017; Tacchi et al., 2016). The cleaved products of the P97/P102 paralogues families and P159 on the cell surface interact with the host and function as receptors for heparin, plasminogen and fibronectin (Bogema et al., 2012; Seymour et al., 2012; Simionatto et al., 2013). Recently, actin was demonstrated extracellularly on porcine epithelial monolayers (PK-15) and it was shown that *M. hyopneumoniae* binds to the extracellular actin exposed on the surface of these cells. Furthermore, P97 possesses two distinct actin-binding regions, suggesting that actin may be an important receptor for *M. hyopneumoniae* (Raymond et al., 2018). Other glycoproteins and cell surface features are probably also involved in binding of *M. hyopneumoniae* to the cilia (Chen et al., 1992; Zielinski and Ross, 1992). Many genes coding for the adhesins contain one or more variable number of tandem repeats (VNTR) regions. The C-terminal portion of P97, identified as R1 region, is responsible for cilium binding. In order for the R1 region to have binding capacity, it needs to contain at least eight R1 repeating units. Variation in number of repeats influences binding capacity (Minion et al., 2000). The P146 is an adhesion lipoprotein and is also extensively processed on the surface of *M. hypopneumoniae* (Bogema et al., 2012). It contains serine repeats, the number of which can vary between strains. Both P97 and P146 repeats are used for differentiation between *M. hyopneumoniae* strains (Mayor et al., 2007; Vranckx et al., 2011). The proteins P97 and P102 are able to recruit plasminogen and fibronectin to the surface of *M. hyopneumoniae*. Bound plasminogen on surface proteins of *M. hyopneumoniae* facilitates conversion to plasmin (Seymour et al., 2012). This process has consequences for tissue invasion and systemic infection (Bogema et al., 2012), suggesting that the adhesins may play a role in virulence (Simionatto et al., 2013). After adhesion to the cilia, the affected cilia clump together and ciliostasis is induced (DeBey and Ross, 1994) and the final stage of the infection is characterized by a marked destruction of the ciliated epithelium, with exfoliation of the epithelial cells (Jaques et al., 1992). This results in impairment of the function of the mucociliary apparatus. In this way and together with the immunosuppressive effects of *M. hyopneumoniae* (discussed further), an efficient clearance of debris and invading pathogens from the upper respiratory tract fails. Pigs infected with *M. hyopneumoniae* are therefore more susceptible to infection with other respiratory pathogenic bacteria such as *Pasteurella multocida (P. multocida)*, *Streptococcus suis (S. suis)*, *Haemophilus parasuis (H. parasuis)*, *Actinobacillus pleuropneumoniae (A. pleuropneumoniae)*, and others. These bacteria are consequently able to proliferate in the respiratory tract. Infection with *M. hyopneumoniae* as triggering factor for establishment of other pathogens is known as EP (Simionatto et al., 2013; Thacker and Minion, 2012).

Production of hydrogen peroxide from glycerol was designated, as a virulence mechanism of certain other mycoplasmas such as *Mycoplasma mycoides* subspecies *mycoides* or *Mycoplasma pneumoniae*. Ferrarini et al. (2018) demonstrated that virulent strains of *M. hyopneumoniae* are able to produce the toxic metabolite hydrogen peroxide from glycerol. Another metabolic difference, partially explaining the different level of virulence was

demonstrated: in comparison with *M. hyorhinis* and *M. flocculare*, a commensal bacterium, only *M. hyopneumoniae* was able to assimilate myo-inositol, and remained viable when this was the primary energy source.

Immune responses are important in preventing infections, although in *M. hyopneumoniae*-infected pigs, the immune response persists and is an important cause of inflammatory lesions. The pig is unable to clear the infection, resulting in a chronic colonisation of the airways by *M. hyopneumoniae* (Razin et al., 1998). It was demonstrated by Pieters et al., (2009) that *M. hyopneumoniae* can persist up to 214 days in the respiratory tract of the pig and total clearance was only demonstrated at 254 days post-infection. It is known that *M. hyopneumoniae* is able to alter the innate and adaptive immune response (Thacker, 2001), although the exact mechanism on how this immunopathological modification arises, is not yet fully elucidated (Thacker and Minion, 2012). After the adhesion step, the peribronchiolar and perivascular connective tissue is infiltrated by macrophages, dendritic cells and B and T lymphocytes (Livingston et al., 1972; Sarradell et al., 2003). However, *M. hyopneumoniae* is able to render the phagocytotic capacity of the macrophages and neutrophils less effective (Asai et al., 1996; Caruso and Ross, 1990) which results in a reduced clearance of *M. hyopneumoniae* and a chronic colonization (Thacker and Minion, 2012). *Mycoplasma hyopneumoniae* induces macrophages also to produce pro-inflammatory cytokines. Normally, cytokines help in the elimination of invading pathogens by stimulating and regulating the innate immune response (Medzhitov and Janeway, 1997; Wooley, 2014). However a sustained production of cytokines, which is the case in *M. hyopneumoniae* infection, leads to host-mediated tissue injury in the lung (Choi et al., 2006; Thacker and Minion, 2012). The next step is chronic inflammation and lymphoid hyperplasia and this seems to be more important for the appearance of lung lesions than the pathogen itself (Thacker and Minion, 2012; Wooley, 2014). This massive infiltration of mononuclear cells (lymphocytes and macrophages) around the blood vessels, peribronchial and peribronchiolar areas and alveolar septa can cause obliteration of the soft-walled bronchioli and collapse of the alveoli (Baskerville, 1981; Sarradell et al., 2003). Next to influencing the macrophage function, it is suggested that *M. hyopneumoniae* is capable of causing a general immune suppressive effect, as the pathogen alters the function of B and T-lymphocytes (Thacker and Minion, 2012). It was demonstrated that *M. hyopneumoniae* cell membranes reduced lymphocyte transformation in response to a T-cell mitogen suggesting a suppression of the cell-mediated immune response (Kishima and Ross, 1985). T-cell dependent mechanisms may play a significant role in the development of pneumonia as thymectomized pigs or pigs injected with anti-thymocyte serum before infection developed less severe microscopic lesions compared to immunocompetent pigs. T-lymphocytes may as well be important to prevent systemic spread of *M. hyopneumoniae*, as in one thymectomized pig from the same study *M. hyopneumoniae* was isolated in the spleen (Tajima and Nei, 1984).

## *1.3. Epidemiology*

*Mycoplasma hyopneumoniae* is ubiquitously present throughout all swine producing countries in the world (Hillen et al., 2014; Thacker and Minion, 2012). Although the highest infection levels are found during the grower-finishing stage, positive pigs detected with nested Polymerase Chain Reaction (nPCR) can be found throughout all stages of pig production (Sibila et al., 2009; Villarreal et al., 2010). An overview of the occurrence of *M. hyopneumoniae* in pigs of different ages is given by Villarreal et al., (2010).

*Mycoplasma hyopneumoniae* can make its entrance in a herd via purchase of subclinically affected replacement gilts or other pigs (Sibila et al., 2009). Once established in the herd, the pathogen can be transmitted from the dam to their piglets via nose-to-nose contact (Desrosiers, 2001; Thacker and Minion, 2012). Gilts or low parity sows are the main transmitters, as they have less *M. hyopneumoniae*-specific immunity and therefore excrete more microorganisms than older sows (Lowe, 2012; Maes et al., 1996). Once this vertical transmission has occurred, further horizontal transmission, independent of age can take place between pen mates or between pigs from adjacent pens if no solid partitions between the pens are used (Maes et al., 1996; Piffer and Ross, 1984). The spread of *M. hyopneumoniae* in a stable is slow (Maes et al., 1996). Meyns et al, (2004) showed that one infected pig may infect a penmate within a time span of six weeks. Another route of entrance in the herd is via airborne transmission from other infected farms. The distance to neighbouring farms to avoid airborne transmission should probably be at least three km (Goodwin, 1985; Muirhead and Alexander, 1997), however this distance was set before it was demonstrated that *M. hyopneumoniae* can be transmitted over a distance of 4.7 km (Dee et al., 2009) and 9.2 km (Otake et al., 2010). No intra-uterine transmission, nor lactogenic transmission has been described (Prikazsky, 1988). The role of fomites is probably not important when standard biosecurity protocols are followed by personnel (Batista et al., 2004). Transmission via semen is unlikely to be a route of transmission as well. There is only one report available where *M. hyopneumoniae* could be isolated from one out of 101 semen samples, but it is not yet clear whether infected semen can result in infection (Schulman and Estola, 1974).

## *1.4. Clinical signs and lung lesions*

Epizootic *Mycoplasma hyopneumoniae* disease is uncommon and occurs when *M. hyopneumoniae* enters an immunological naïve herd. In this case, morbidity may reach 100%, acute respiratory distress, coughing, pyrexia and even death may occur.

Within two to five months, the infection evolves in the form most commonly observed namely endemic mycoplasmosis (Thacker and Minion, 2012). The characteristic dry, non-productive cough appears slowly in the herd, peaks four weeks after initial infection, and may last for eight weeks. Non-complicated *M. hyopneumoniae*-disease, although rarely seen, may take a sub-clinical course, or dry non-productive coughing may be present, next to slight fever and anorexia (Del Pozo Sacristán, 2014; Maes et al., 1996). Involvement of other respiratory agents will aggravate the clinical picture: productive cough, fever, anorexia, laboured breathing or thumping, prostration, higher morbidity and even mortality can be seen. Reduced feed intake will result in uneven growth and lower daily weight gain (Maes et al., 1996; Thacker and Minion, 2012). Clinical signs of EP are usually not observed in piglets younger than six weeks of age (Sibila et al., 2009), however in some cases animals of three to four weeks may start coughing (Maes et al., 1996).

Catarrhal pneumonia is found in the apical, cardiac, accessory lobe(s) and the ventral portions of the diaphragmatic lobe in non-complicated *M. hyopneumoniae* infections (Maes et al., 1996; Thacker and Minion 2012). The lesions have a meaty consistency and are red to purple. On the cut surface of such an uncomplicated lesion, a catarrhal exudate is visible (Thacker and Minion, 2012). These lesions appear from seven days onwards post infection, reach a maximum size in four weeks post-infection (Kobish et al., 1993) and recovery occurs after seven to ten weeks. Instead, red to purplish or white interlobular scar retractions of connective tissue, called

fissures, appear (Del Pozo Sacristán, 2014; Kobish et al., 1993; Thacker and Minion, 2012; Whittlestone, 1972). Lung lesions originating from *M. hyopneumoniae*-infections, complicated by secondary pyogenic pathogens, affect a larger portion of the lung, have a more greyish and firm appearance, a mucopurulent exudate is seen in the airways and more time is needed to resolve (Osborne et al., 1981; Thacker and Minion, 2012).

Although the lung lesions observed in complicated and non-complicated *M. hyopneumoniae* infections are suggestive for EP, they are not pathognomonic. It is well known that other pathogens, especially viral respiratory agents such as influenza, can cause similar macroscopic lesions (Sibila et al., 2009; Thacker, 2001).

## *1.5. Parameters to assess the severity of Mycoplasma hyopneumoniae infections in combination with confirmation of the presence of the bacterium*

Assessing the severity of a *M. hyopneumoniae* infection is necessary to compare the virulence of different *M. hyopneumoniae* strains and it is an important tool to evaluate the efficacy of vaccines or vaccination strategies. Below, an overview is given of the main parameters used to assess this severity. It is recommended to use a combination of methods to estimate the severity of a *M. hyopneumoniae*-infection and it is necessary to confirm the presence of infection with *M. hyopneumoniae* in the investigated pigs, preferably by PCR.

### 1.5.1. Respiratory disease score

Coughing is the principal symptom of respiratory disease and it is stated that it is the main clinical sign of EP (Kobish and Friis, 1996; Maes et al., 1996; Nathues et al., 2012; Ross, 1992). Coughing can be caused by many infectious agents, although a dry non-productive cough is a typical symptom of EP, especially in finishing pigs. When presence of *M. hyopneumoniae* can be confirmed in the same herd with other diagnostic procedures, such as PCR, assessing the occurrence of coughing might be a useful parameter to assess the severity of EP as the likelihood of a high coughing index was increased with 76% in herds with ≥50% of the bronchoalveolar lavage fluids positive for *M. hyopneumoniae* (Nathues et al., 2012). Leon et al. (2001) found that in fattening pigs of farrow-to-finishing farms, coughing started around the same time as when these pigs seroconverted to *M. hyopneumoniae*.

Several techniques to record coughing in pigs have been described. One technique determines the number of coughing incidents per pig per minute during a total period of three minutes in rest, without arousing the pigs (Mombarg et al., 2002; Straw, 1986). Other techniques describe the number of coughing bouts per pig after encouraged move. In the coughing index from Bahnson (Bahnson, 1993; Nathues, 2011; Nathues et al., 2012), a pen or two neighbouring pens within a compartment with at least 20 pigs is/are selected. Subsequently, the pigs are forced to move by shouting and clapping and onsets of coughing are counted for a period of three minutes. Multiple coughing bouts of the same pig are included in the scoring if coughing was absent for 10 seconds before reappearing of the next cough. The same procedure as above is repeated in the same pigs one minute later and next the whole procedure of two times three minutes is repeated with a second batch of at least 20 pigs at another location in the same compartment, but in a different pen/adjacent pens. The average coughing index is

subsequently calculated as the number of coughing bouts divided by the actual number of pigs observed, multiplied by the minutes of observation time. The coughing index from Bahnson, gives the percentage of pigs coughing within one minute after encouraging them to move (Bahnson, 1993; Nathues, 2011; Nathues et al., 2012). A fairly comparable coughing score was applied by Mateussen et al., (2001). The pigs were observed for 10 minutes after an arousal period of 2 minutes. The number of pigs that coughed is counted and is divided by the total number of pigs present in the pen. This number was subsequently multiplied by 100 to obtain the coughing index. A combination of counting the coughs in rest and after arousal was proposed by Halbur et al., (1996). This scoring system requires to be performed daily by the same person, always at the same moment and preferably in the morning as scoring in rest is facilitated before the pigs start to be active. This scoring system ranges from 0-6 with 0 (no coughing), 1 (mild coughing after encouraged move), 2 (mild coughing in rest), 3 (moderate coughing after encouraged move), 4 (moderate coughing in rest), 5 (severe coughing after encouraged move), 6 (severe coughing in rest) and daily averages are calculated per group. This approach gives a detailed image on the course of the respiratory disease over time. However, it is more suitable for pigs in small groups, i.e. under experimental circumstances as the pigs need to be individually identified and are allowed to have one score per day (the highest score). Automatic recordings of coughing events have been developed, not only to obtain a coughing score, which can be useful in larger farms, but also to identify the pens in a stable where the coughing index is higher. Identifying the pens with (the highest) coughing might be useful to limit antimicrobial use, as not all pigs from all pens need to be treated, (Exadaktylos et al., 2008; Silva et al., 2008). An example of this is the Pig Cough Monitor (PCM), which performs a continuous (24 hours per day, seven days a week), automated measurement of porcine respiratory health through sound analysis. The microphone is mounted at a height of two meters in the centre of a pig compartment. The software is based on an algorithm to recognise cough sounds from other non-coughs, i.e. environmental sounds, screams of the pigs,…. The advantage of the automated systems compared to the discrete cough counts, is that a continuous measurement of coughing in the pig compartments can be performed and an identification of the pens with coughing pigs is possible as well, however this requires multiple microphones (Hemeryck et al., 2015; Silva et al., 2008). The disadvantage is that the device needs to withstand the harsh conditions in the pig stable without interfering with the sound acquisition itself: i.e. unstable power supplies, high temperatures and humidity, acid compounds in the air, internet connection problems, accelerated corrosion due to ammonia concentrations, rats biting cables, etc. (Hemeryck et al., 2015).

### 1.5.2. Macroscopic lung lesions

The severity or extent of macroscopic *Mycoplasma*-like lesions or the characteristic cranioventral pulmonary consolidation can be determined by visualisation and palpation of the lung in a two or three-dimensional approach (Garcia-Morante et al., 2016). The two-dimensional approach determines the estimated proportion of lung surface affected by *Mycoplasma*-like lesions and is used in the scoring systems of Goodwin et al. (1969), Hannan et al. (1982), Madec and Kobisch (1982), Straw et al. (1986) and Sibila et al. (2014). Goodwin et al. (1969), Hannan et al. (1982) and Madec and Kobisch (1982) express the severity of the lesions by means of a points system instead of percentage (Sibila et al., 2014; Straw et al., 1986) to indicate the affected lung fields. The

more precise three-dimensional approach determines the proportion of affected lung area and multiplies this by the relative weight each lobe represents in the total lung weight and expresses the severity of the lesion as the weight percentage of affected tissue. However, the relative weight for each lobe can differ between methods. Following scoring systems use the three-dimensional scoring approach: Morrison et al. (1985), Christensen et al. (1999) and the reference system of the European Pharmacopoeia (Garcia-Morante et al., 2016; Christensen et al., 1999; Morrison et al., 1985; Pharmacopoeia, 2013). A complete overview of the most used scoring systems to determine the severity of *Mycoplasma*-like lesions, is given by Garcia-Morante et al. (2016). The same authors concluded that all frequently used scoring systems to determine macroscopic lung lesions in *M. hyopneumoniae* infected pigs were very compatible, except for one method based on image analysis. This could be explained by the fact that the latter method only quantified the extent of the lesions from images of the dorsal surface of the lung and lesions of the accessory lobe were not considered in the image analysis. If both surfaces of the lung would have been included in the image analysis, the correlations with the other methods might have been improved (Garcia-Morante et al., 2016). By this objective scoring system, a dorsal picture of a lung is taken and analysed by means of an online free software tool. The ventral side of the lung is pictured only when the lesion is present on that side. The area affected by lesions and total area of the dorsal side of the lung are delineated in each picture in order to calculate the percentage of affected lung by the following formula: (right lung affected area + left lung affected area)/total lung area*100 (Sibila et al., 2014). Other methods based on image analysis have been developed (Ross et al., 1984; Asai et al., 1993 and Murphy et al., 1993), however the method of Sibila et al. (2014) might be preferred above these other methods, as it is the most recent method developed and because of the availability of the free software tool.

### 1.5.3. Microscopic lung lesions

The severity of broncho-interstitial pneumonia is often scored according to the degree of peribronchiolar and perivascular lymphohystiocytic infiltration, nodule formation and presence of exudate in the airways using light microscopy on hematoxylin and eosin stained formalin-fixed, paraffin-embedded tissue sections. Different scoring systems have been developed with small adaptations between the scoring systems. The scoring system of Livingston (1972) classifies the histological lesions as follows: - : indicates negative or no lesions; + indicates one or more lymphoid nodules involving the muscularis mucosae of bronchi and bronchioles; ++ indicates lymphoid nodules affecting the muscularis mucosae of bronchi and bronchioles and the presence of inflammatory cells in the septal wall, bronchi, and alveolar lumen; +++ indicates perivascular and peribronchiolar hyperplasia of the lymphoid tissue with inflammatory cells in the alveolar septa and neutrophils in the bronchial and alveolar lumens; and ++++ indicates massive perivascular and peribronchiolar lymphoid hyperplasia in extended areas of the lung parenchyma. The scoring system of Morris et al. (1995), ranges from 1-5 with 1) limited infiltration of macrophages and lymphocytes around bronchioles, with airways and alveolar spaces free of cellular exudates; score 2) light to moderate infiltrates with mild diffuse cellular exudates into airways; score 3, score 4 and score 5, respectively mild (score 3), moderate (score 4) and severe (score 5) lesions characteristic of broncho-interstitial pneumonia, centered around bronchioles but extending to the interstitium, with lymphofollicular infiltration and mixed inflammatory cell exudates. Scores 1 and 2 are considered not to be related with a *M. hyopneumoniae*

infection, while scores 3 to 5 are presumptive of a *M. hyopneumoniae* infection. The scoring system of Calsamiglia et al. (2000) is an adaptation of both scoring systems of Livingston et al. (1972) and Morris et al. (1995), and in the scoring system of Opriessnig et al. (2004) the lung sections are scored for presence and severity of type 2 pneumocyte hypertrophy and hyperplasia, alveolar septal infiltration with inflammatory cells, peribronchial lymphoid hyperplasia, amount of alveolar exudate, and amount of inflammation in the lamina propria of bronchi and bronchioles ranging from 0 to 6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate multifocal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse). The downside to these above mentioned scoring systems is that they are semi-quantitative and subjective.

The severity of pneumonia can also be measured objectively with image analysis. Using this method, a digital microscopic image is taken from a hematoxylin and eosin stained, formalin-fixed, paraffin-embedded lung tissue section. The percentage of air in this lung section is subsequently measured by means of automated image analysis software, which calculates the percentage of air in each lung field. The obtained percentage is inversely proportional to the lymphohistiocytic infiltration in the lung tissue, the amount of atelectasis, and the intrabronchiolar and bronchial exudate, intra-alveolar exudate and/or oedema and proliferation of type II pneumocytes (Del Pozo Sacristán, 2014), and therefore a good objective way to measure the severity of pneumonia. The obtained results in challenged infected pigs with a *M. hyopneumoniae* isolate obtained from pigs experiencing a clinical course of EP were 39.3, 41.6, 48.5 and 47.3 % for four different isolates. In the pigs in the negative control group a percentage of 52.2 was detected (Vicca et al., 2003).

### 1.5.4. Number of *Mycoplasma hyopneumoniae* organisms in the respiratory tract

The number of organisms in the respiratory tract of the pig may also reflect the severity of the disease (Vranckx et al., 2012a). The number of organisms colonizing the lungs of the pig probably depends on the accumulation of infection doses, the capacity of the strain itself to multiply in the lung and time after infection (Thacker and Minion, 2012). Detection of antigen of *M. hyopneumoniae* can be performed by immunohistochemistry or immunofluorescence. Kobish et al. (1978) developed a scoring system ranging from 0 to 3 with 0: no immunofluorescense (IF), 1: limited IF, 2: moderate IF and 3: intense IF (Del Pozo Sacristán, 2014; Kobisch et al., 1978; Vicca et al., 2003). The limitation of this technique is that it is time-consuming, laborious, the amount of *M. hyopneumoniae* antigen is determined in a semi-quantitative way and subjective (Del Pozo Sacristán, 2014). A more accurate assay that allows the detection of the pathogen and the quantification of *M. hyopneumoniae* is quantitative real-time Polymerase Chain Reaction (qPCR) (Dubosson et al., 2004; Marois et al., 2010; Strait et al., 2008a; Strait et al., 2008b). The number of *M. hyopneumoniae*-organisms also depends on the sampling site in the respiratory tract. In pigs pre-mortal tracheobronchial swabbing or bronchoalveolar lavage is more sensitive compared to oropharyngeal brushing and nasal swabbing (Fablet et al., 2010). This is consistent with the fact that trachea and bronchi are the multiplication sites of *M. hyopneumoniae* (Blanchard et al., 1992). Laryngeal swabs (swabbing the internal walls of the laryngeal cartilages) of 8-week old pigs after experimental *M. hyopneumoniae* infection was demonstrated to be a reliable sampling technique from five days post-inoculation as well: it showed the highest sensitivity compared to nasal swabs and bronchoalveolar fluids, while oral fluids had the lowest sensitivity of all these samples (Pieters et al., 2017). Lung tissue or flushing at the main bronchus/bronchi is

suitable, but is only possible post mortem and variable results are reported for detection of *M. hyopneumoniae* DNA in lung tissue (Sibila et al., 2009).

## *1.6. Factors influencing the severity of the disease*

### 1.6.1. Virulence and pathogenicity of *Mycoplasma hyopneumoniae* strains

In some farms, control measures, such as improving management practices, antimicrobial therapy and implementing vaccination against *M. hyopneumoniae* do not result in the expected level of effect against *M. hyopneumoniae* infections, whereas in other farms, these measures are sufficiently effective. Vicca et al. (2002) found that in farms with clinical problems of *M. hyopneumoniae* but with good management and housing conditions, compared to farms with subclinical problems and poor management and housing, the pigs in the first category of farms had higher seroprevalences against *M. hyopneumoniae* and that they were infected earlier on during the production cycle. They concluded that apart from housing and management, additional factors such as differences among *M. hyopneumoniae* isolates may determine the outcome of the disease. Indeed, *M. hyopneumoniae* isolates from different swine herds, may have a high variation in virulence, with highly virulent strains being able to induce more and more quickly severe pneumonia lesions in a larger proportion of pigs than low virulent strains (Meyns et al., 2007; Vicca et al., 2003; Villarreal et al., 2011). The higher virulence of these strains is attributed to the capacity to adhere or better adhere to the cilia of the respiratory tract, a higher ability to multiply in the lungs and to induce a more severe inflammation (Meyns et al., 2007; Tajima and Yagihashi, 1982; Young et al., 2000). Little is known about specific virulence factors or factors attributable to these differences observed in *M. hyopneumoniae* isolates, and why the most closely related porcine *Mycoplasma* species, *Mycoplasma flocculare* (*M. flocculare*) is a commensal of the pig and does not cause disease (Siqueira et al., 2013). Nevertheless, the whole genome of four *M. hyopneumoniae* strains has been sequenced completely and should theoretically facilitate this process of identifying possible virulence factors or factors related to pathogenicity (Liu et al., 2011; Minion et al., 2004; Vasconcelos et al., 2005) by intensively comparing the genome, transcriptome, proteome, metabolome and secretome of these strains (Ferrarini et al., 2016; Liu et al., 2013; Maes et al., 2017; Paes et al., 2017; Pinto et al., 2009; Siqueira et al., 2014; Siqueira et al., 2013). Vicca et al. (2003) could correlate the presence of a 5000 bp fragment with *M. hyopneumoniae* virulence in a small number of isolates, using RAPD analysis, however more isolates need to be investigated and a specific function could not be attributed to this fragment. Some evidence is available that the cell envelop of *M. hyopneumoniae* plays a role in the virulence (Meyns et al., 2007). A thinner capsule enveloping the *Mycoplasma* cells induced by *in vitro* passage, demonstrated by Tajima and Yagahashi, (1982) by ruthenium red staining was associated with a decreased pathogenicity. Differences in the composition of glycoproteins and fatty acids of the cell membrane were detected when avirulent *M. hyopneumoniae* strains were compared with virulent strains. Virulent strains were able to induce higher cytoplasmic calcium concentrations in neutrophils, compared to non-virulent strains, suggesting altering of the neutrophil signal transduction mechanisms by virulent *M. hyopneumoniae* strains (Chen et al., 1992). This was confirmed by Park et al. (2002), who found that pathogenic strains of *M. hyopneumoniae* were capable of increasing intracellular calcium concentrations in porcine ciliated epithelial cells, while this was not observed in non-

pathogenic *M. hyopneumoniae* strains and *M. flocculare* strains. They concluded that this intracellular increase of calcium may serve as a signal for inducing loss of cilia.

Passaging of the *M. hyopneumoniae* strain *in vitro* or *in vivo* may alter its virulence quickly: DeBey and Ross (1994) showed that after 20 to 40 *in vitro* passages compared to only 1 or 2 passage(s), *M. hyopneumoniae* strain 232 had a lower capacity to induce ciliar damage. However, when the same strain was passaged in gnotobiotic pigs *in vivo*, the strain was reversed to virulence (DeBey and Ross, 1994; Hannan et al., 1984). Another example of this passaging effect is the J-strain, used to date in many commercially available *M. hyopneumoniae* vaccines. Upon isolation the strain was virulent causing mild pneumonia in sows, but after continued *in vitro* passaging, the J-strain lost its capacity to adhere, and also its capacity to cause disease in pigs (Mebus and Underdahl, 1977; Thomas et al., 2003; Zielinski and Ross, 1993). A different expression level of genes, like those encoding adhesins can have an impact on virulence, as Pinto et al. (2009) compared the expression of proteins of two different virulent strains (strain 7448 and 7422) and the J-strain. They concluded that in the virulent strains an overexpression of 64 proteins was observed, including an overexpression of the P97 adhesin, while this was not the case in the non-virulent J-strain. The same author found a 23 kb region similar as a previously described Integrative Conjugal Element of *Mycoplasma fermentans* (ICEF) in the genetic material of virulent strains 7448 and 232 of *M. hyopneumoniae*, that was absent in J-strain (Pinto et al., 2007). This element is generally recognized to be involved in virulence and coding sequences related to ICEF were detected in *Mycoplasma bovis* and *Mycoplasma agalactiae*. However, the function in *M. hyopneumoniae* remains unclear (Maes et al., 2017; Pinto et al., 2007). This element was also found in the virulent strain 168, but not in its attenuated variant (Liu et al., 2013). The fact that these putative ICEH's were observed in three virulent *M. hyopneumoniae* strains, but not in a non-virulent strain, might imply that potentially mobile genetic elements involved in lateral gene transfer are implicated in virulence of *M. hyopneumoniae*, and this might be a tool to obtain genome variability (Maes et al., 2017; Pinto et al., 2009; Pinto et al., 2007). Siqueira et al. (2013) compared the genome organisation of *M. flocculare* and *M. hyopneumoniae*, and found that although the similarity between these species is high, the genes encoding proteins involved in host-cell adhesion differ in genomic structure and organisation: some genes encoding adhesins of the P97 family are absent in *M. flocculare*, contain sequence differences or lack domains which are important for adhesion. However, they were not able to find specific virulence determinant factors that could explain the difference in virulence between both investigated species (Siqueira et al., 2013). Paes et al. (2017) compared the secretomes of *M. hyopneumoniae* and the commensal *M. flocculare*. They found that a higher number of proteins were secreted by the virulent *M. hyopneumoniae* (62) compared to *M. flocculare* (26). Overall, 15 virulence-related proteins were found in *M. hyopneumoniae* and only four in *M. flocculare*, of which two proteins were shared with *M. hyopneumoniae*. Taken together, more research is needed to find clear-cut factors that enable us to make the differentiation between virulent and non-virulent *M. hyopneumoniae*-strains or to find markers which determines why *M. hyopneumoniae* is a virulent pathogen and *M. flocculare* a commensal (Maes et al., 2017).

## 1.6.2. Diversity of *Mycoplasma hyopneumoniae* strains

*Mycoplasma* spp. are the smallest known self-replicating organisms (Artiushin and Minion, 1996). It is believed that they attained their small genomes through degenerative evolution from a Gram-positive ancestor, losing a lot of important metabolic pathways such as those involved in phospholipid, amino acid and purine and pyrimidine synthesis and obtaining a reduced number of tRNAs (Maniloff, 2002; Muto and Ushida, 2002). This has made the organism highly susceptible to environmental conditions, but also dependent on the host for growth and survival (Strait et al., 2008a). *Mycoplasma hyopneumoniae* has a genome size of 892 kilobase pairs and because of the reduced genome the organism needs to rely on diversity to cope with the adverse environment of the host (Madsen et al., 2007; Minion et al., 2004).

Antigenic variability in *M. hyopneumoniae* has been demonstrated (Assunção et al., 2005; Calus et al., 2007; Wise and Kim, 1987; Zhang et al., 1995). All members of the P97/P102 paralogue family are cleaved to a greater or lesser extent and this depends on the isolate (Burnett et al., 2006; Djordjevic et al., 2004). The presence of different adhesion related proteins with multiple binding sites is believed to prevent the elimination of the organism from the host in the presence of an adhesion-specific immune response, resulting in chronic infections (Hsu and Minion, 1998). Calus et al., (2007) found that the protein variability of 56 *M. hyopneumoniae* isolates from six different countries and 37 different herds was high. A protein variability of 25% was found between the investigated field isolates and when comparing the field isolates to the J-strain a protein variability of 30% was observed. However, they could not find differences between isolates originating from the same herds. Assuncao et al. (2005) on the other hand found that the protein patterns of 18 *M. hyopneumoniae* field isolates (Gran-Canaria) were very homogenous and Scarman et al. (1997) found that the protein profiles from five out of six isolates of different geographical regions (Australia, UK, USA and Canada) were very homogenous as well.

Genetic diversity of *M. hyopneumoniae* was demonstrated by Frey et al. (1992) and further confirmed by Arthiusin and Minion (1996), Kokotovic et al. (1999), Djordevic et al. (2004), Mayor et al. (2007; 2008), Madsen et al. (2007) Stakenborg et al. (2005; 2006), Tamiozzo et al. (2011), Nathues et al. (2011), Vrankx et al. (2011; 2012c), Charlebois et al. (2014), Dos Santos et al. (2015), Burgher Pulgarón et al. (2015), Tamiozzo et al. (2015), Pantoja et al. (2016) and Takeuti et al. (2017). Several methods have been used to type *M. hyopneumoniae*, including Pulsed-Field Gel Electrophoresis (PGFE) (Stakenborg et al., 2005), Amplified Fragment Length Polymorphism (AFLP), RAPD or also called arbitrarily primed PCR (Artiushin and Minion, 1996; Stakenborg et al., 2006), microarray technology (Madsen et al., 2007), PCR-Random fragment length polymorphism (PCR-RFLP) (Stakenborg et al., 2006), sequencing of a single locus (polyserine repeat motif of P146) (Mayor et al., 2007), Multiple-Locus Sequence Typing (MLST) (Mayor et al., 2008) and Multiple-Locus of variable number of tandem repeats (MLVA) (Burgher Pulgarón et al., 2015; Charlebois et al., 2014; Dos Santos et al., 2015; Nathues et al., 2011; Pantoja et al., 2016; Takeuti et al., 2017; Tamiozzo et al., 2015; Vranckx et al., 2011; Vranckx et al., 2012c). An overview of different molecular typing methods is shown by Vranckx et al. (2012b). Next-generation sequencing can be added to these techniques. Vranckx et al. (2011) and Nathues et al. (2011) were the first to investigate the use of MLVA for typing *M. hyopneumoniae* strains. This technique amplifies specific regions in the genome of *M. hyopneumoniae*. Typically, regions prone to slipped strand mispairing or homological recombination events are

chosen, because these events cause these regions to be highly variable in different *M. hyopneumoniae* strains. Such regions containing a repeat sequence in the genome of *M. hyopneumoniae* are called variable number of tandem repeats (VNTRs) (Torres-Cruz and van der Woude, 2003). These VNTRs are without exceptions translationally in frame, and code for variable number of amino acid repeats (VNTARs). These VNTARs code for putative structural, physicochemical and antigenic variations in the corresponding proteins, with potential implications regarding the pathogenicity of *M. hyopneumoniae* (de Castro et al., 2006). For instance, many regions in the genome of *M. hyopneumoniae* related to adherence in the host contain VNTRs. Recombination events and slipped strand mispairing of these VNTRs, will possibly lead to expression of a different sized protein and might have implications in the adhesion function of the pathogen (Torres-Cruz and van der Woude, 2003; Vranckx et al., 2012b). The more divergent two strains are, the more VNTR regions in these strains will contain a different number of repeats. The length of these regions can subsequently be determined with gel or capillary electrophoresis after amplification of the VNTRs with PCR (Vranckx et al., 2012b). Vrankx et al. (2011) concluded that this technique is relatively inexpensive compared to other typing methods, has a high discriminatory power, is quick and reliable, can be used on bronchoalveolar lavage fluid (BALF) samples and tracheobronchial swabs, and is able to differentiate *M. hyopneumoniae* strains, without prior cultivation of the pathogen. This was confirmed by other authors (Charlebois et al., 2014; Dos Santos et al., 2015; Nathues et al., 2011). Table 1 shows the results of different studies using MLVA in *M. hyopneumoniae* research.

**Table 1a: Overview of different studies using MLVA to type *Mycoplasma hyopneumoniae* strains**

| Amplified region of *M. hyopneumoniae* genome | Number and origin of *M. hyopneumoniae* samples | Results | Reference |
|---|---|---|---|
| P97R1<br>P146R3<br>H1<br>H5R2 | -42 reference field isolates, different origin<br>-45 samples, BALF or tracheal swabs<br>3 herds, Belgium | -herd 1: one strain<br>-herd 2: one strain at 10 w. of age, 3 from 20 w. onwards, pigs with 2 strains detected<br>-herd 3: two strains detected at 10, 15, 20 w. of age, not at 26 w. and again a different strain detected at the next two sampling points of 26 w.<br>→ different strains between herds, pigs can be colonized with multiple strains | (Vranckx et al., 2011) |
| P97R1<br>P146R3<br>H4 | -19 reference field isolates, different origin<br>-109 herds, 52 isolates from lung tissue or BALF, 45 pigs in Germany | Separate analysis of the loci:<br>-8 different P97 lengths<br>-6 different P146 lengths<br>-9 different H4 lengths<br>→ herds and individual pigs were colonised by different strains | (Nathues et al., 2011) |
| P97R1, P146R3<br>H1, H5R2 | 4 herds, 20 BALF samples per herd at 6, 10, 14 and 26 w., Belgium | -one distinct strain is mainly present in one herd, persisting for at least 12 w.<br>-in herd 1 and 3: 3 and 2 clonal variants | (Vranckx et al., 2012c) |
| Locus 1*, Locus 2*<br>P97R1, P97R2<br>*No further information | 239 isolates from pigs with (n=210) or without (n=29) pneumonia, France, 5 reference strains | -129 different MLVA-patterns detected, different patterns from pigs originating from the same farm, different patterns in the same pig | (Marois-Créhan et al., 2012) |
| Locus 1[1], Locus 2[2]<br>P97R1, P97R2<br>[1]18 bp repeat region<br>[2]12 bp repeat region | 160 lungs, 48 farms from 2 slaughter houses, Canada | -87 MLVA-types detected<br>→ high diversity, greater homogeneity within farms<br>-strains missing a specific MLVA-locus: significant less lung lesions | (Charlebois et al., 2014) |
| P97R1<br>P146R1 | -335 *M. hyopneumoniae* positive PCR samples<br>*Brazil (n=95), bronchial swabs<br>*Mexico (n=26), bronchial swabs<br>*Spain (n=25), bacterial isolates<br>*USA (n=209) bronchial and nasal swabs | -139 MLVA-types detected<br>→multiple variants *of M. hyopneumoniae* are circulating in swine<br>-P97: 17 different types (2-18 repeats)<br>-P146: 34 different types (7-48 repeats), > 30 repeats in the samples in Brazil and Spain, while shorter repeats were detected in USA and Mexico | (Dos Santos et al., 2015) |
| P97R1<br>P146R3<br>H2R1<br>H4 | -15 nasal swabs from pigs with respiratory signs<br>-98 tracheal swabs and 6 BALFs from pneumonic lungs<br>36 BALFs from non-pneumonic lungs, 5 farms, samples taken at a slaughter house, Cuba | Separate analysis of the loci:<br>-8 genotypes of P97<br>-14 genotypes of P146R3<br>-20 genotypes of H2R1<br>-15 genotypes of H4<br>→ a high genetic variability was detected in healthy and pneumonic lungs<br>→ Strains having 3 VNTR copy numbers in P97R1, were detected only in pneumonic lungs and strains having 40 and 43 VNTR copy number in P146R3 were detected only in apparently healthy lungs | (Burgher Pulgarón et al., 2015) |

**Table 1b: Overview of different studies using MLVA to type *Mycoplasma hyopneumoniae* strains**

| Amplified region of *M. hyopneumoniae* genome | Number and origin of *M. hyopneumoniae* samples | Results | Reference |
|---|---|---|---|
| P146R1<br>P146R3<br>H4<br>H5<br>P95 | -6 commercial bacterins<br>-28 BALF at slaughter, from 3 herds, Argentina | Separate analysis of the loci:<br>-different genetic *M. hyopneumoniae* subtypes among and within herds<br>-analysis of the herd samples:<br>→P146R1: 1 type (a)<br>→P146R3: 7 types (e, f, g, h, I, j, k)<br>→P95: 2 types (m, n)<br>→H4: 3 types (o, p, q)<br>→H5: 4 types (t, u, v, w)<br>-analysis of the commercial bacterins<br>→P146R1: 4 types (a, b, c, d), bacterin B, 3 types for this allele : b,c,d<br>→P146R3: 2 types (j, l)<br>→P95: 1 type (m)<br>→H4: 3 types (q, r, m)<br>→H5: 1 type (u) | (Tamiozzo et al., 2015) |
| P97<br>P146 | -105 samples from 3 nursery-to-finish farms, USA<br>-BALF from coughing pigs<br>-TBS from on-farm mortalities | -95 samples were typable<br>-4 MLVA-variants, 3 clusters<br>→9-15: 61 samples<br>→11-21: 26 samples<br>→9-21: 1 sample<br>→7-15: 7 samples | (Pantoja et al., 2016) |
| P97R1<br>P146R3 | 103 samples from 3 farms,<br>Minas Gerais, Santa Catarina, and Rio Grande do Sul, Brazil | -17 different *M. hyopneumoniae* variants<br>-locus P97R1: 5 types (3-12 repeats)<br>-locus P146R3: 11 types (14-45 repeats)<br>-variant 3-17 and 3-24 detected in 2 out of 3 farms (farm A and B)<br>→6 variants detected in farm A<br>→6 variants detected in farm B<br>→2 variants detected in farm C | (Takeuti et al., 2017) |
| P97<br>P146 | USA, different states, 3 year period<br>4 production flows<br>-within each flow 1-3 farms per production stage (gilt developer, sow farm, nursery, finisher)<br>-total number of samples obtained not mentioned | -1 production flow: one MLVA-type across all four production flows<br>-remaining 3 production flows: 1-4 MLVA-types, MLVA types varied by 1-2 VNTRs for each locus | (Fano et al., 2018) |

BALF: bronchoalveolar lavage fluid, w.: weeks of age, bp: base pair, TBS: tracheobronchial swabs, VNTR: variable number of tandem repeats

It is clear from the table that no consistency is present in reporting *M. hyopneumoniae* –diversity data, and that different authors use different terminology to report *M. hyopneumoniae* diversity. From the table it can be concluded as well that a high genetic diversity is seen in *M. hyopneumoniae* in specific countries/regions and that different pigs can harbour different strains of *M. hyopneumoniae*. However, comparing data from different studies is not straightforward. There may be differences in reporting the data, amplifying a different number of VNTR's, using different sample types, selecting different types of herds, laboratory differences (running specifications of the gel or capillary gel electrophoresis) and differences in interpretation of the electropherograms. One must therefore be very careful in comparing results between different studies and extrapolation of results obtained in one region/country to the situation in another region/country. Only limited research has been conducted in investigating strain diversity in different production rounds within the same herds. The impact of strain variability on lung lesions or the severity of the disease is still not well defined (Maes et al., 2017). Although some studies point in the direction that co-infection with more than one strain might result in more severe lung lesions (Villarreal et al., 2009; Vranckx et al., 2011), other studies did not find such a relationship and investigation on a larger scale with more samples and following up consecutive batches is needed (Charlebois et al., 2014; Maes et al., 2017). The fact that the pathogenesis of *M. hyopneumoniae* is not fully elucidated and no virulence markers have been found, complicates investigating the impact of a particular strain on the severity of lung lesions and clinical signs. The finding that several *M. hyopneumoniae* strains are co-existing in one individual pig complicates this even further (Dos Santos et al., 2015; Maes et al., 2017; Nathues et al., 2011; Vranckx et al., 2011).

Field isolate variability of *M. hyopneumoniae* can impede the proper diagnostics of the pathogen. Genetic differences may express proteins on the cell surface of *M. hyopneumoniae* to a different extent. Five different *M. hyopneumoniae* isolates (232, OOMP1502, OOMP1301, 95MP1505, 95MP1509), were used to challenge infect seven pigs each and sera of the pigs were tested with three *M. hyopneumoniae* specific ELISAs: the Tween 20 ELISA, the HerdChek® *M. hyopneumoniae* ELISA (IDEXX Laboratories, Westbrook, ME), both indirect assays detecting numerous antigens associated with the cell membrane of *M. hyopneumoniae* and the IDEIA™ *M. hyopneumoniae* EIA KIT (Oxoid, Ely, Cambridgeshire, UK, formerly DAKO™ *M. hyopneumoniae* ELISA), a competitive-inhibition ELISA designed to detect a conserved epitope of the *Mycoplasma hyopneumoniae* 74KDa protein. All pigs except one were positive with the Oxoid™ ELISA 28 days post challenge and fewer positives were detected with the IDEXX and Tween 20 ELISA. In case of the OOMP1502 up to four (IDEXX) or five (Tween 20) negative test results were obtained, when the same pigs tested positive with the Oxoid™ ELISA. A possible explanation might be that antibodies produced by certain isolates might less effectively bind to the antigens used in the IDEXX and Tween 20 ELISA or that for certain isolates less antibodies are produced by the pig (Strait et al., 2008a). In case of detection of *M. hyopneumoniae* DNA of different strains, the same conclusion was made by Strait et al. (2008b): submitting 36 *M. hyopneumoniae* isolates to a panel of published *M. hyopneumoniae*-specific PCR assays resulted in five assays not detecting every *M. hyopneumoniae* isolate of the collection. These five assays targeted the following three genes: a putative ABC transporter (GenBank accession no. U02537) (Dubosson et al., 2004; Verdin et al., 2000); a repeated element (REP; GenBank accession no. AF004388) (Dubosson et al., 2004; Stärk et al., 1998); a target that spanned the hypothetical genes mhp023 and mhp024 (GenBank accession no. AE017332; base pairs 27057 to 28020) (Kurth et al., 2002).

### 1.6.3. Husbandry characteristics

Improving management practices is primordial in the control of *M. hyopneumoniae* infections and should be addressed first before implementing other measures (Maes et al., 2008). Herd size is a commonly known factor to influence the risk for introduction of respiratory agents in a farm (Stärk, 2000). This was demonstrated by Aalund et al. (1976), who found a gradual increase for the risk on respiratory disease with increasing number of pigs produced. On the other hand, owners of larger herds are more likely to adopt management and housing practices which may supersede this increased risk, compared to owners of smaller herds (Gardner et al., 2002). Frequently purchasing pigs is a risk factor significantly associated with a higher prevalence of pneumonia (Meyns et al., 2011).

Farrow-to-finish farms who purchase gilts have a higher percentage of pigs seropositive for *M. hyopneumoniae* (Maes et al., 2000). An unstable parity distribution is likely unfavourable as gilts or low parity sows have low antibody titers and excrete more *Mycoplasma* organisms than older sows (Fano et al., 2006; Maes et al., 1996). Multiple source purchase policy is positively associated with respiratory disease (Hurnik et al., 1994) and purchasing a lot of animals at one occasion has been described as a risk as well, as the higher the number of pigs purchased, the higher the probability of buying in one infected animal (Rosendal and Mitchell, 1983). Purchasing of pigs is inherently connected to the herd type: fattening farms are obliged to buy their pigs, and the chance for a multi-source purchase policy is likely higher compared to breeding farms (Stärk, 2000).

Continuous pig flow is disadvantageous to reduce respiratory disease compared to production systems where all pigs are moved in batches (all-in/all-out). All-in/all-out production is probably the main management factor in controlling EP, and is considered even more important than herd size (Clark et al., 1991; Flesjå and Solberg, 1981; Maes et al., 2008; Stärk, 2000). This production system allows the farmer to clean, disinfect and implement a downtime period between groups of pigs (Maes et al., 2008).

Decreasing stocking density in different production stages has been shown to have a positive impact on respiratory disease (Byrt et al., 1985). Flesja et al. (1982) showed that > 12 pigs per pen (an indicator of stocking density), can have a negative influence on respiratory disease. Overcrowding makes the pig more susceptible to disease by causing stress and improving the circumstances to transmit pathogens, which in case of *M. hyopneumoniae* enhances the chance to transmit the pathogen via nose to nose contact (Maes et al., 2008; Thacker and Minion, 2012). Lindquist (1974) found that respiratory diseases are more prevalent in farms with ≥ 500 pigs in one section, <3 $m^3$ air space and <0.7$m^2$ area per pig.

Prevention of *M. hyopneumoniae* spread within herds or facilities can be done by maintaining basic hygienic principles for rearing pigs (Amass and Clark, 1999) and limiting factors that may destabilize herd immunity, such as parasite control and avoidance of infections with other respiratory agents (Maes et al., 2008). Separate equipment, personnel, feed, water, and transport vehicles should be provided for each production stage (Hege et al., 2002; Román et al., 2006; Stankovic et al., 2010). Rodent and insect control should be implemented, albeit the importance of such measures for the control of *M. hyponeumoniae* is not clear (Maes et al., 2008). Indirect transmission of *M. hyopneumoniae* through fomites has been suggested as transmission route by Goodwin (1985), however when standard biosecurity measures are considered by farm personnel weekly in contact with infected

pigs, *M. hyopneumoniae* was not transmitted to naïve pigs during a 20 week period (Batista et al., 2004; Goodwin, 1985). In fact, a one-night downtime period of approximately 14 hours prevents the spread of *M. hyopneumoniae*, next to Porcine Reproductive and Respiratory Syndrome virus (PRRSv) by personnel and fomites (Pitkin et al., 2011).

Infectious aerosols, containing microorganisms such as *M. hyopneumoniae*, and generated by coughing, sneezing and simple exhalation, may be a source of infection to neighbouring farms as Goodwin et al. (1985) and Zhuang et al. (2002) found that the risk of a herd becoming infected is inversely related to the vicinity to other farms. The pig density in the area (Rose and Madec, 2002; Zhuang et al., 2002), close proximity of finishing farms (Hege et al., 2002) and the presence of *M. hyopneumoniae* infected farms (Goodwin, 1985; Hege et al., 2002; Stärk et al., 1992) are factors closely associated with the risk of a herd becoming infected with *M. hyopneumoniae*. Hege et al. (2002) found that parking sites for pig transport in an area of ≤ 200 m from the farm is a potential source of introduction of respiratory pathogens in the farm. It has been shown that airborne transport of *M. hyopneumoniae* is possible through a distance of 9 km (Dee et al., 2009; Otake et al., 2010), although other authors state that to theoretically avoid airborne transmission, the minimum distance should be at least three km (Zhuang et al., 2002).

From point of view of reducing transmission of *M. hyopneumoniae*, it is preferred to wean the piglets between one and three weeks of age (Maes et al., 2008) although weaning before the age of 21 days in pigs is not allowed in the EU (European Council, 2008).

Sows infected in the 200 days prior to farrowing are likely to transmit *M. hyopneumoniae* to their offspring. Gilts are most likely to be infected within the 200 days prior to farrowing. The number of pigs infected at weaning depends on the number of sows shedding *M. hyopneumoniae* at farrowing and this in turn determines the disease load from *M. hyopneumoniae* in growing pigs (Lowe, 2012). Therefore, an adequate gilt acclimation program should focus on reduction of *M. hyopneumoniae* shedding at first farrowing (Garza-Moreno et al., 2016). In Europe and North-America, vaccination of replacement gilts is the main tool for gilt acclimation, however, there is a trend in the US nowadays of deliberately exposing the gilts to the pathogen (Garza-Moreno et al., 2018). It was already demonstrated that the absence of gilt acclimation by providing the replacement stock access to other living animals was addressed being a risk factor for *M. hyopneumoniae* disease severity in fattening pigs (Garza-Moreno et al., 2016; Nathues et al., 2016). Additionally, with the knowledge that gilts even after adaptation and after their first progeny are crucial in the spread of *M. hyopneumoniae* in the herd, in larger production systems they are housed separately from the herd until they reach second gestation. By that time, these second parity sows have acquired the desired immunity level in order not to destabilize the herd anymore (Hoy et al., 1986; Joo, 2003; Maes et al., 2008).

## 1.6.4. Housing conditions with emphasis on indoor climate

### 1.6.4.1. Particulates

A fattener pig breathes about 40 kg of air per day, compared to consuming 2.7 kg of feed and drinking 4 kg of water (Gonyou et al., 2006). Over the last decades, pig husbandry has become more intensive, leading to higher concentrations of airborne particulates (Pedersen et al., 2000). Not only particulates are of major concern to evaluate air quality, but gasses (see further) may not be neglected, both as an animal and human health hazard (Gonyou et al., 2006).

The term PM can be defined as a complex mixture of suspended particles with different biological, chemical and physical properties. In this mixture, particles of different shape, size, density and chemical composition can be found (Cambra-López, 2010). The term PM is mainly used in the context of air quality and is in this definition referred to as fine liquid or solid particles suspended in a gaseous medium (Ulens, 2015). In atmospheric science the latter is mostly referred to as aerosol (Cambra-López, 2010). The behaviour of particles in the atmosphere or respiratory tract is described by the scientific term aerodynamic equivalent diameter (AED). This term groups three of the particle properties described above in one single parameter: shape, size and density. It is defined as the diameter of a spherical particle with a density of 1 g/cm$^3$ with the same settling velocity under gravity as the particle in question (Cambra-López, 2010; Kulkarni et al., 2011; Ulens, 2015). In this thesis, only the size-selective classification of PM, $PM_{10}$, $PM_{2.5}$, $PM_1$ is used, although the different fractions of PM can be defined in multiple ways (Ulens, 2015). $PM_{10}$, $PM_{2.5}$ and $PM_1$ can therefore be defined as particles that pass through a size-selective inlet with a 50% cut-off at respectively 10 μm, 2.5 μm and 1 μm AED (U.S. EPA, 2004; Ulens, 2015). PM can as well be grouped based on its origin or source. Primary PM originates from mechanical processes and is emitted directly into the atmosphere (Ulens, 2015). Primary PM can also contain bacteria or parts of bacteria (Banhazi et al., 2004; Curtis, 1972; Hinz, 2002), fungal spores (Harry, 1978; Noble et al., 1963), moulds and their specific mycotoxins and viruses such as rotavirus and PRRSv. The organic matter in larger particles is able to protect micro-organisms against adverse physical and chemical factors, such as exposure to changes in humidity, sunlight, oxygen etc. (Butera et al., 1991; Harry, 1978). Secondary, PM on the other hand, originates from chemical reactions between gasses (ammonia, nitrogen oxide,…) and particles in the atmosphere (Ulens, 2015). Toxic gasses are able to attach to the porous surface of particulate matter (Harry, 1978; Hinz, 2002). For instance ammonia can be carried on PM for an extended period of time (Kaasik and Maasikmets, 2013). Odours can be adsorbed to particulates as well (Takai and Pedersen, 2002). For PM to become and stay airborne the attraction between the particle and the surrounding air molecules has to be larger than the force of gravity on the particle (Collins and Algers, 1986).

*A. Characteristics of particulates*

The naked human eye can only see particles larger than 50 μm in a ray of sunlight. Therefore, the air in a pig facility can be loaded with more (smaller) particles than thought at first sight. The ability of a particle to be deposited in the respiratory tract depends on its size (Collins and Algers, 1986). Following particle size ranges are

often distinguished: inhalable or total dust or also referred to as all particles of all sizes suspended in the air or expressed in size all particles smaller than 50-100 μm, (Gonyou et al., 2006), thoracal dust or fine dust (particles smaller than 10 μm) will reach the higher respiratory airways, and respirable dust (particles smaller than 4 μm, in the past often defined up to 5μm), will reach the respiratory part of the lung (Hinz, 2002; Jacobson et al., 2003; Takai et al., 1998). Particulate matter originating from housed livestock, also referred to as bio-aerosols, contain more particulates with biological activity and/or origin compared with urban and industrial PM (Cambra-López et al., 2010). The smaller the size of the particulates, the deeper they can penetrate into the lungs and the more harmful they are (Aarnink and Ellen, 2007). Particles with a size larger than approximately 5 μm will be trapped by the mucociliary system (Collins and Algers, 1986). On the other hand, Harry (1978) indicated that although smaller infected particles can be transported directly to their target area i.e. the trachea, bronchi or lungs (diseases of the upper respiratory tract), the larger particles could be more infective as they are more likely to carry a larger amount of microorganisms.

Particulate matter in animal production can originate from different sources. Viable or non-viable microorganisms such as fungi, viruses, bacteria or parts of their cell wall (Banhazi et al., 2004; Curtis, 1972; Hinz, 2002), feed and building materials in the facilities inside animal facilities (Hinz, 2002; Lemay et al., 2002), skin cells, hair from the animal species housed in the stable, dried manure, urine, grain mites, spores, pollen, soil, mineral additives in pig feed and insect parts may be added to the list of sources contributing to PM formation (Hinz, 2002; Kim et al., 2008). Only a small portion of PM encountered in the facility will enter via the incoming ventilation air (Mostafa and Buescher, 2011). Of bacteria detected in the air of a swine facility, Gram-positive organisms such as *Staphylococcus* spp., *Micrococcus* spp., *Aerococcus* spp., *Enterococcus durans*, *Streptococcus suis*, *Bacillus* spp. and *Corynebacterium* spp. can be detected. Of the Gram-negative bacteria *Enterobacter agglomerans*, *Acinetobacter calcoaceticus*, *Pasteurella* spp. and *Vibrio* spp. were detected (Gonyou et al., 2006). Following fungi have been detected in swine building indoor environments: *Aspergillus versicolor* was the most frequent species found in air (21%), followed by *Scopulariopsis brevicaulis* (17%) and *Penicillium sp.* (14%). Other genera were as well isolated: *Cladosporium* sp., *Fusarium* sp., *Mucor* sp., *Trichoderma* sp., *Phoma* sp., *Alternaria* sp., *Chrysosporium* sp., *Scytalidium* sp., *Pithomyces* sp., *Acremonium* sp., *Chrysonilia* sp, *Hormographiella* sp., *Ochroconis* sp., *Beauveria* sp., *Botrytis* sp., *Stachybotrys* sp., *Epicoccum* sp., *Lictheimia* sp., *Staphilotrichum* sp., *Ulocladium* sp., *Stemphilium* sp. and *Rhizopus* sp. (Viegas et al., 2013). In a review article regarding mycotoxins as indoor air contaminants, it is stated that exposure to five important agricultural mycotoxins deoxynivalenol, fumonisins, aflatoxins and zearalenone under normal circumstances is very unlikely. Allergic responses (asthma, allergic rhinitis, hypersensitivity pneumonitis) are the most common problems associated with inhalation exposure to fungi, the extent of the effect depending on the health status of the exposed person or animal (Jarvis and Miller, 2005). Antibiotics, used in the feed, can also be detected in PM. Up to five different antibiotics (oxytetracycline, chlortetracycline, sulfamethazine, tylosin and chloramphenicol) were detected in sedimentation PM samples from fattening pigs collected from 1981 to 2000 (Hamscher et al., 2003). Dried manure can also be a source of antibiotics detected in PM. Tetracyclines and sulphonamides are poorly metabolized in pigs, thus significant amounts of the parent drug can therefore be excreted (Hamscher et al., 2003; Winckler and Grafe, 2001). There might be a risk to develop antibiotic

resistance upon inhaling PM contaminated with antibiotics as this may represent a permanent exposure to sub-therapeutic concentrations (Hamscher et al., 2003).

*B. Particulate matter measuring methods*

Particulate matter concentrations may be measured with different methods. Most commonly used equipment is a tapered element oscillating microbalance, a beta attenuation monitor, a gravimetric sampler and an optical particle counter (Ulens, 205). The latter device was used in this thesis because of the demonstrated equivalence according to EN 13205 for this particular spectrometer (Grimm 1.109 spectrometer), which describes a European standardized procedure to assess the performance of PM sampling with different sampling techniques in a workplace environment and the equivalence of this device with the Reference Sampler for measuring $PM_{10}$ and $PM_{2.5}$ in ambient air (EN 12341, 1999; EN 14907, 2005; Van Ransbeeck et al., 2013b). Optical particle counters individually detect particles in aerosol by the principle of light scattering by the particles inside the optical measuring cell of the device (Van Ransbeeck et al., 2012). The intensity of light scattering correlates with a certain particle size (Van Ransbeeck et al., 2013a). Based on the intensity of the detected signal, the signal is subsequently classified into size channels (Ulens, 2015). Conversion of the counts to PM mass density is performed and by means of the software of the devices used, $PM_{10}$, $PM_{2.5}$ and $PM_1$ mass fractions are calculated (Van Ransbeeck et al., 2013a). More information regarding different measuring equipment can be consulted in Ulens (2015) and Van Ransbeeck et al. (2013b).

*C. Concentration and size distribution of particulates in pig farm*

In a study of Takai et al. (1998) the mean inhalable and respirable PM concentrations were 2.19 and 0.23 $mg/m^3$, respectively. In general terms, the total PM mass concentration in swine confinement buildings in various sections of the barn and over the year will stay between 2.0 and 3.0 $mg/m^3$ (Lemay et al., 2002). The recommended maximal concentration of PM estimated on the basis of dose-response correlation to swine health or human health problems is 2.4 $mg/m^3$ (Donham, 1991).

*D. Impact of particulates on pig health*

The impact of PM on the respiratory tract can be described as mechanical, chemical, infectious, immunosuppressive, allergic and toxic (Hartung, 2002). Thus it may compromise health and performance of livestock in different ways: as a carrier of pathogenic and non-pathogenic micro-organisms, as an allergic or immunosuppressive agent, as a physical irritant of the respiratory tract and as a carrier of toxic chemicals and odors (Baekbo, 1990; Carpenter and Moulsley, 1986; Donham, 1991; Hartung and Schulz, 2011; Hinz, 2002).
Endotoxin is probably the most relevant parameter identified with respirable particulates and associated with lung function impairment (Millner, 2009). Endotoxins are lipopolysaccharide complexes originating from the outer membrane of Gram-negative bacteria (Cambra-López et al., 2010). They are released into the environment during bacterial cell growth and after cell death (Spaan et al., 2006) by handling of the bedding material or animal feed (Zucker et al., 2000). The highest amount of endotoxin in the air is detected in weaner houses and during the day, probably due to the diurnal rhythm in animal activities (Seedorf et al., 1998). It originates mostly from

Gram-negative, obligate anaerobic bacteria, and in minority from *Escherichia coli* present in faeces or manure or from bacteria of the species *Enterobacter agglomerans* (epiphytic organism on a variety of plants) (Zucker et al., 2000). Under normal circumstances the pigs' respiratory tract is not free of infection, or even lesion, but it is usually free of disease. Environmental factors, like PM can tip the balance between host and respiratory invaders in favour of the invaders (Donham, 1991). Particulates can have an inhibitory effect on the mucosal clearance system. Endotoxins in particulates originating from bacteria or the particulates themselves, can act as a chemical component causing inflammation. The inflammation process can damage the nasal turbinates which can diminish the removal of particulates (Pearson and Sharples, 1995). The airway epithelial cells, alveolar macrophages and neutrophils are over activated, which can lead to a loss of protective effect of alveolar macrophages and other lung defence mechanisms (Jolie et al., 1999). As *M. hyopneumoniae* is known to act on the mucosal clearance system as well, by disrupting the cilia on the epithelial surface, particulates (and ammonia, see further) and the bacterium may work in a synergistic way (Thacker, 2006). Inhalation of infectious aerosol by susceptible animals is a route of transmission in a number of viral and bacterial diseases. For instance *M. hyopneumoniae* was detected in the air by means of a nested PCR assay (Stärk, 1999) and acute and chronic exposure of pigs to aerial pollutants has been implicated in the aetiology of multifactorial respiratory diseases (Wathes et al., 2004). Particulates combined with ammonia take part in the pathogenesis of progressive atrophic rhinitis (Hamilton et al., 1999; Robertson et al., 1990) and the incidence and severity of specific respiratory diseases, such as EP in the weaned pig are higher when combined with chronic exposure to aerial pollutants (Demmers et al., 2003; Wathes et al., 2004). It has been demonstrated as well that even non-pathogenic organisms, when inhaled in a very high concentration can be harmful for the health of the pig (Carpenter and Moulsley, 1986). Pigs exposed to aerial pollutants may have a reduced performance (Demmers et al., 2003). Some authors have doubts about the deleterious effect of PM on pig health and performance (Done, 1991; Takai et al., 1998). An overview of a selection of relevant literature of the effect of aerial pollutants is provided in table 2.

**Table 2a: Results of a selection of relevant literature of the impact of aerial pollutants on health and production parameters**

| Indoor pollutant | Duration exposure | Age (or kg) | Effect on health parameters | Effect on production parameters | E / F | Reference |
|---|---|---|---|---|---|---|
| **Control**:<br>**PM + $NH_3$**: 50 ppm + 300 mg/$m^3$ | 57d | 8.2 kg | Turbinates, trachea and lungs normal | effect on ADG ($P<0.1$)<br>treatment: 420 g/pig/day<br>Control: 520 g/pig/day | E | (Curtis et al., 1975) |
| **Monthly collection environmental parameters $NH_3$ and BCFP during 1 year**<br>-farrowing unit and nursery of 4 farms | 1 year | Weight at onset nursery not mentioned, 140 d<br>average weight<br>Farm A: 76.4<br>Farm B: 71.4<br>Farm C: 69.0<br>Farm D: 71.7 kg | No apparent effect of $NH_3$ and BCFP on health parameters (coughing, sneezing) | No apparent effect of $NH_3$ and BCFP on performance data (weight) | F | (Underdahl et al., 1982) |
| **Environmental parameters** :<br>**Correlation** of total and respirable PM, endotoxin, total and respirable bacteria, total fungi, $NH_3$ and $CO_2$, collected 10 months preceding production and health data<br>29 farms, 19 finishing and 10 farrowing buildings | Environ mental data collected for 60 days | Age finishers not specified | No significant correlation between measured environmental data in farrowing unit and health data<br>Significant correlation<br>-total PM and ascarid scar<br>-endotoxin and ascarid scar<br>-ammonia and arthritis<br>-ammonia and porcine stress<br>-ammonia and abcesses<br>-respirable and total microbes and pneumonia<br>-Total microbes and pneumonia<br>-Total microbes and abcesses<br>-Respirable PM with pneumonia, pleuritis, and arthritis<br>-endotoxin with arthritis<br>-ammonia (120 cm above floor) with arthritis<br>-ammonia (20 cm) with pneumonia, pleurisy, arthritis<br>-carbondioxide (120 cm above floor) with pleurisy, arthritis | Farrowing/ nursery<br>Significant correlation between<br>-total PM and age at 25 kg of BW<br>-respirable PM and age at 25 kg of BW<br>-ammonia with pigs weaned per litter<br>-bacteria (CFU/$m^3$ with kg of feed/25 kg of pig weight, age at 25 kg body weight, pigs weaned per litter, pig mortality<br>-Fungi (CFU/$m^3$) with , age at 25 kg BW, pig mortality<br>-total microbes (count/$m^3$) with kg of feed/25 kg of pig, age at 25 kg BW,<br>-respirable bacteria (count/$m^3$) with kg of feed/25 kg of BW, age at 25 kg of BW<br>Finishing:<br>Significant correlation<br>-Total PM (mg/$m^3$) with excess death loss<br>-Respirable PM death loss<br>-carbondioxide (120 cm above floor) death loss | F | (Donham, 1991) |

Table 2b: Results of a selection of relevant literature of the impact of aerial pollutants on health and production parameters

| Indoor pollutant | Duration exposure | Age (or kg) | Effect on health parameters | Effect on production parameters | E / F | Reference |
|---|---|---|---|---|---|---|
| **Study 1:**<br>**FR** (room with filtered air)<br>**NFR** (room without air filtration)<br>7 replicates<br>**Study 2:**<br>**VC**: Room + vacuum cleaning<br>**C**: Room without vacuum cleaning<br>2 replicates<br>**Study 3:**<br>**WA**: Room with weekly washing of pigs WA<br>**NWA**: Room not weekly washing of pigs, 6 replicates | Study 1: 6 wk<br>Study 2: finishing period, wk not mentioned, (24 kg-107 kg)<br>Study 3: not mentioned | Study 1: 4 wk (7.5 kg)<br>Study 2: 10 wk (24 kgs)<br>Study 3: 4 wk (7.5 kg) | Study 1:<br>-% lung lesions higher in FR*<br>-% pleurisy higher in NFR *<br>Study 2:<br>more veterinary treatments ($p<0.05$) and ↑lung lesions in C*<br>Study 3:<br>-no significant difference in veterinary treatments between groups<br><br>*not significant | Study 1<br>Study 2:<br>Study 3:<br>-no significant difference between groups<br>ADG, ADFI, mortality | F | (Van 't Klooster et al., 1993) |
| **FARM:** one week old pigs remained on a conventional farm until 20 wk<br>**ISO:** pigs from the conventional farm weaned at 2 wk and kept in isolated research facilities until 20 wk with lower inhalable + respirable PM peptidoglycan and endotoxin, $NH_3$, $CO_2$ compared to FARM | 19 wk (FARM)<br>18 wk (ISO) | 1 wk (FARM)<br>2 wk (ISO) | ↑ macroscopic lung lesions indicative for *M. hyopneumoniae* in pigs from FARM compared to pigs raised in ISO | ADG (5-20 wk) significantly ↑ in pigs from ISO compared to pigs from FARM | F + E | (Jolie et al., 1999) |

E: experimental study, F: field study, %: percentage, d: days, ADG: average daily gain, BCFP: bacterial colony forming particles, BW: body weight, wk: weeks old

*E. Impact of particulates on human health in swine farmers*

From human health perspective dust exposure in pig farming is important because of the large number of workers needed and the increasing number of working hours inside enclosed buildings in pig production. Symptoms most commonly noticed are chronic bronchitis (cough and phlegm), asthma-like symptoms such as wheezing and shortness of breath during work, with evidence of mild airways obstruction (Iversen et al., 2000). A clear dose-response relationship with the number of working hours inside swine facilities and respiratory symptoms has been demonstrated (Radon et al., 2000).

*F. Influencing factors on the concentration of PM in pig stables*

F.1. Type of farm and animal characteristics

The concentration of PM in pig stables depends on the species, age of the animals, stocking density, feeding and littering strategies, climate conditions and behaviour of the animals (Hinz, 2002). Winkel et al. (2015) measured indoor concentrations $PM_{10}$ and $PM_{2.5}$ in poultry houses, pig facilities and dairy farms. Inside concentrations were highest in poultry houses, followed by pig facilities and the lowest concentrations for each of these fractions measured were detected in dairy houses. The PM-concentration increases with the age of the pigs, and consequently is lower in post-weaning facilities than in finishing units (Pedersen et al., 2000). Close to the time of weaning, PM-concentration is also much higher than before farrowing (Kim et al., 2005). In farrowing units faeces take a larger part in the constitution of PM-formation, compared to feed (Donham, 1991), while for weaner pigs the PM seemed to originate mainly from feed and skin particles (Aarnink and Ellen, 2007). In growing-finishing pigs a similar contribution of feed, faeces and skin particles was found (Aarnink and van der Hoek, 2004). The amount of PM is influenced by the activity of the animals (Dawson, 1990). Animal activity causes local disturbance of settled PM (Carpenter, 1986). The overall particulate levels will generally be higher during the day than overnight because of more animal activity during the day (Kim et al., 2005; Pedersen et al., 2000). The number of sows in a farm - as an indicator of farm size - is positively correlated to respirable particle concentration during summer according to Banhazi et al. (2004; 2008). A linear relationship exists between the total weight of the pigs in a room and the PM-concentration: this concentration more than doubled during a 16-week finishing period. Therefore the animal mass present in the building will be a major factor influencing the PM-concentration (Pedersen et al., 2000).

F.2. Housing

Animals kept in total confinement are more exposed to PM compared to animals kept in open byre types or buildings with space boarding or another large permanent opening. The type of floor, presence of bedding material/litter, size of the air space, and to a much lesser extent the building materials of the pig facility are all able to influence the constitution of PM (Banhazi et al., 2008; Dawson, 1990). In pig facilities with slatted floors, PM levels are much lower compared to the ones with solid floors: the area for PM to settle is much lower and this minimizes resuspension of PM from animal activities (Dawson, 1990; Jacobson et al., 2003). Presence of bedding and agitation significantly increases the amount of PM in the air space of the pig. Inhalable particle

concentrations increase with the size of the air space (building) in all-in/all-out production systems and decrease in continuous flow systems (Banhazi et al., 2008).

F.3. Feed

The composition of PM and its particle size distribution largely depends upon the type of feed and feeding method. The addition of fat or oil to the feed has been shown to reduce both aerial and settled dust levels: adding 1 or 2% of fat, reduces the PM with 10 or 20%, respectively (Dawson, 1990). According to Honey and McQuitty (1979), the feeding activity is the main cause of airborne dust in swine houses. Restricted feeding twice a day is recommended instead of feeding *ad libitum* to decrease the PM content in the pigs' air space. This is related to the fact that when restricted feeding is applied, the activity of the pigs is more synchronized (Aarnink and Ellen, 2007). Using a pig feeder should be preferred above feeding the pigs on the floor (Dawson, 1990). Supplying liquid feed lowers the PM-concentration compared to dry feed. Providing pelleted feed to nursery pigs instead of a meal diet, significantly increased $PM_{10}$, $PM_{2.5}$ and $PM_1$ (Ulens et al., 2015). However, the quality of the pelleted feed probably plays an important role in PM production as well: PM-formation is determined by how easily the pellets disintegrate during transport (Aarnink and Ellen, 2007) and coated pellets lead to lower PM-formation than non-coated pellets (Aarnink and Ellen, 2007; Dawson, 1990). Some ingredients cause higher PM-production than others: barley, for instance, causes more PM-formation than corn (Aarnink and Ellen, 2007). The feed distribution mechanisms and spilling feed can increase the PM-levels in a swine facility (Hartung, 1986; Jacobson et al., 2003). Feed delivered by a screwing system causes 150% more dust than feed delivered by hand in the trough. In automatic feeding systems, a substantial amount of particulates can become airborne, when feed drops into the feeder or trough (Aarnink and Ellen, 2007).

F.4. Season and climatological influence

Season or climate, intrinsically associated with ventilation rates, will affect PM concentrations inside a swine facility (Cambra-López, 2010). Banhazi et al. (2008) measured significantly higher inhalable particle concentrations in swine buildings during winter than during summer. Higher ventilation rates during summer will diminish PM concentrations, lower ventilation rates in winter will result in higher PM concentrations (Cambra-López, 2010). Aerial level of lung-deposal BCFP is higher during cold weather (Curtis et al., 1975; Jacobson et al., 2003).

F.5. Thermal environment

Temperature is positively correlated with respirable particle concentration in all-in/all-out buildings and in weaner, deep-bedded shelter and grower/finisher buildings, but was negatively correlated with particle concentrations in the farrowing unit and other sow rooms (Banhazi et al., 2008). Kim et al. (2005) found a positive correlation between the indoor temperature and total particulates. Lower temperatures on the other hand may also stimulate animal activity, which in turn increases the PM content of the facility because the settled particulates on surfaces and floors are resuspended in the air (Collins and Algers, 1986; Kim et al., 2005). The effect of temperature on PM concentration is therefore not easily explained.

F.6. Humidity

When humidity inside a facility is high, the moist content of the settled dust, litter and faeces will be high and may prevent to some degree the amount of airborne particles generated (Collins and Algers, 1986; Pedersen et al., 2000). In fact, to reduce airborne particulates, the optimal absolute humidity should be as high as possible, without creating condense and animal discomfort. Smaller particulates clump together by the high humidity. Hence the settling velocity of such a particle will be increased (Harry, 1978). When the relative humidity exceeds 80%, the infection pressure rises and a higher prevalence of several diseases will be noticed. Relative humidity below 60%, particularly at low temperatures, results in an increased PM content in the air (Harry, 1978). Kim et al. (2005) found that the trend of correlation between indoor relative humidity and environmental risk factors (total dust, total airborne bacteria, $NH_3$, $H_2S$, odour index level) was generally negative.

E.7. Husbandry activity

Feeding and handling the pigs, as well as bedding promote the generation of particulates (Dawson, 1990). Therefore it is better to reduce the visits by stockmen. Passages between the pens are preferred to reduce stock disturbance (Harry, 1978) .

F.8. Hygiene

Cleaning has a positive effect on the amount of settled particulates on smooth surfaces. Residues may persist on difficult accessible areas (i.e. roof beam, wall structures, ventilators) (Harry, 1978). Interestingly, Banhazi et al. (2004) found that in deep-bedded shelters the effect of pen soiling had a beneficial effect. It was hypothesized that soiling would increase humidity inside the building (see above) and that the smaller range particles can be trapped in the adhesive layer of dirt. However, in conventional weaner and grower/finisher buildings with (partially) slatted floors, pen soiling was associated with a higher respirable particle concentration. For cleaning the facilities, hosing and pressure washing are preferred above scraping, but pressure washing seemed only marginally better than hosing (Cargill et al., 2002). The use of disinfectants will have a positive effect as well, especially on cracked old floors, however as many disinfectants are inactivated by organic material, thorough cleaning should be the first step. On the other hand Ulens et al. (2014) showed that an additional wet cleaning and disinfection step after broom-swiping and vacuum cleaning the pens did not result in consistently lower indoor concentrations of PM (next to $NH_3$, $CO_2$, $CH_4$, $N_2O$). They indicated that a thorough dry-cleaning step might be sufficient and the wet step in the protocol might not be necessary to reduce the aerial pollutants any further.

*G. Particulate mitigation strategies inside the facilities*

Techniques addressing PM formation inside the swine facility are also called source-oriented mitigation techniques. This can be achieved by prevention of PM formation or diminishing the concentration inside the swine facility. This is in contrast with end-of pipe techniques which try to lower the pollutants in the outgoing air, reducing pollutant emission, but do not tackle the formation inside the facilities (Ulens, 2015). More

information on end-of pipe techniques is provided by Ulens (2015). Most source-oriented mitigation strategies were already described under the paragraph influencing factors on the concentration of PM in pig stables, such as type of feed and delivery method, hygiene in the pens, the use of bedding materials. Additionally, spraying oil or water has been shown to reduce indoor PM concentrations (Aarnink and Ellen, 2007). According to Takai and Pedersen (1999) the droplets should be >150 μm for the reduction to be effective and to avoid that smaller droplets might be inhaled by the pigs and pig workers. Applying an oil film on the pigs is also a possibility (Osman et al., 1999). An increased ventilation rate during short periods of time might dilute and thus reduce total PM concentrations. This effect however, only lasts for a short amount of time (Robertson, 1989). Filtration of the inside air (internal circulation dry filters, electrostatic precipitation, ionisations systems) is able to lower inside PM concentrations. However, due to the large volume housing systems and the high costs of the devices, this method is impractical in swine buildings (Ulens, 2015).

### 1.6.4.2. Indoor gasses

Ammonia ($NH_3$), hydrogen sulphide ($H_2S$), carbon dioxide ($CO_2$) and methane ($CH_4$) are the main pollution gasses in animal houses. They are produced by decomposition of animal waste and the metabolism of the animal (Heber et al., 1997; Muehling, 1970; Ni, 1998; Ni et al., 2008). Gasses such as $NH_3$ and $H_2S$ can be adsorbed on particulates. Their porous structure allows slow and continuous gas release from the particulates over a longer period and enables the toxic gas to reach the deeper part of the lungs, when particulates with adsorbed gas are inhaled (Donham et al., 1977; Takai and Pedersen, 2002).

Ammonia is mostly originating from drying of manure and urine on solid floor surfaces. It is formed through enzymatic hydrolysis of urea by the enzyme urease in faeces and produced by various microorganisms leading to the formation of carbonic acid and ammonia which is in equilibrium with $NH_3$ depending on temperature and pH of the faeces (Ni, 1999; Wenger, 1999; Zeeman, 1999). Volatilisation occurs under the form of $NH_3$ and depends on its concentration in the manure, pH, temperature, surface area and air velocity at the manure surface (Ni, 1999). A lesser portion of $NH_3$ in pig facilities originates from storage of liquid manure in the manure pit (Wenger, 1999). Ammonia can be measured by different techniques: aqueous acid trap, gas detection tubes, Fourier Transform-Infrared Spectroscopy (FTIR), chemiluminescence, infrared photoacoustic system, laser absorption spectroscopy…Most of these techniques for continuous measuring are costly (Ulens, 2015). The technique used in this thesis is the infrared photoacoustic system (Innova photoactoustic gas monitor 1314). Air is drawn into the analysis cell of the measurement system by means of an air pump. Infrared light is reflected off a mirror passing a mechanical chopper, causing the light to pulsate. Next, the light beam passes through one of the optical filters in the filter wheel inside the device. The wavelength of the incoming beam can be modulated by these installed filters. At a certain wavelength, specific for each gas, the infrared light will cause excitation of the gas molecules, which causes an increase in temperature. By chopping the light beam, the temperature will increase and decrease, causing an equivalent increase and decrease in pressure of the monitored gas in the hermetically closed photoacoustic cell. These pressure pulses (an acoustic signal) are detected by microphones in the cell. The acoustic signal is proportional to the concentration of the measured gas (Innova 1314I technical

data sheet). For further reading on this subject, Ulens (2015) may be consulted. Ammonia levels of 5 to 10 ppm are typically encountered in well-ventilated swine confinement buildings with slatted floors. The ammonia concentration tends to be slightly higher in buildings with solid floors (Jacobson et al., 2003). Acute exposure to $NH_3$ can cause damage to the epithelial lining of the less resistant bronchioli, prolonged exposure to concentrations above 400 ppm may destroy mucous membranes by dissolving keratin, fat and cholesterol (Stombaug et al., 1969). For the comfort of the farmer and staff it is recommended that the $NH_3$ levels are kept below 25 ppm, but pigs will already avoid $NH_3$ levels of 10 ppm. The maximum long-term $NH_3$ exposure for swine should be less than 20 ppm as both pathological (Hamilton et al., 1996) and immunological data suggest that $NH_3$ influences the inflammatory response (Urbain et al., 1994). Ammonia levels of 50 to 75 ppm reduced the ability of young pigs to clear bacteria form the lungs (Gonyou et al., 2006). Very high levels of $NH_3$ , (≥2.500 ppm) may be fatal (Jacobson et al., 2003). In 2004 a ministerial decree was passed, requiring all pig and poultry producers in Flanders when renovating, expanding or building new animal facilities to use officially approved low ammonia-emission housing systems (M.B. 19 maart 2004; Ulens et al., 2014). Although these housing systems are mostly built at a higher cost compared to conventional housing systems, Ulens et al. (2014) demonstrated that such a low-ammonia-emission system did not result in a reduction of indoor pollutant concentrations, except for $CH_4$ compared to a conventional housing system for finisher pigs with fully slatted floors. Ammonia can also play a role in the development of turbinate atrophy in pigs affected with dermonecrotic (DNT) producing *Pasteurella multocida (P. multocida)* (Hamilton et al., 1999). It facilitates colonization of DNT *P. multocida* in the upper respiratory tract and thus increases the severity of the clinical disease progressive atrophic rhinitis (Hamilton et al., 1998). It may also influence the growth rate of pigs (Drummond et al., 1980). A significant effect was observed of 50, 100 and 150 ppm $NH_3$ in four week old pigs for an exposure period of four weeks. The pigs in the respective treatment groups gained respectively 12, 30 and 29% less body weight compared to the control group (0 ppm $NH_3$) (Drummond et al., 1980). No impact of different $NH_3$ concentrations and PM concentrations (50 ppm and 10 mg/m³ or 300 mg/m³) was demonstrated on nasal turbinates, trachea and lungs (Curtis et al., 1975). In Donham et al. (1991) a significant correlation in the finishing unit was found between $NH_3$ (23.6 ppm) and pleurisy lesions (Donham, 1991). In a study of Hamilton et al., (1999) the turbinate atrophy due to DNT *P. multocida* was highest in the pigs in the environmental exposure chambers with $NH_3$ (50 ppm) and ovalbumin particulates (20 mg/m³) compared to all other combinations of treatments (ovalbumin PM, $NH_3$, DNT *P. multocida*) (Hamilton et al., 1999). From these data it can be concluded that studies investigating the systematic long term impact of PM and $NH_3$ simultaneously on (respiratory) pig health or production parameters are not available. In humans, $NH_3$ can act as a local irritant of the mucous membranes and the respiratory tract (Cole et al., 2000). Clinical signs observed are sneezing, coughing and salivation (Donham, 2000). Source-oriented mitigation strategies for $NH_3$ can be implemented at the level of the feed provided to the pigs, for instance, lowering the protein in the diet, acidifying the diet and adding more fibre (Aarnink et al., 2008; Kim et al., 2004; Ulens, 2015). Segregation of urine and faeces preventing the contact between urea in urine and the enzyme urease can be applied, for instance with V-shaped pit floors and scrapers. Reducing the emmiting surface of the manure pit and the use of partially slatted floors

can also be applied to diminish $NH_3$ formation inside the pig facility. More information on $NH_3$ mitigation strategies is provided in an excellent overview by Ulens (2015).

Hydrogen sulphide is formed by bacterial sulphate reduction and the decomposition of sulphur-containing organic compounds in manure under anaerobic conditions (Jacobson et al., 2003; Wenger, 1999) and has the distinctive odour of rotten eggs (Holland et al., 2002; Wenger, 1999). Hydrogen sulphide is heavier than air and can accumulate in manure pits, holding tanks, and other low areas in a facility (Holland et al., 2002). It is considered the most dangerous gas in animal facilities and at the level of manure storage as it is responsible for many deaths both in human and livestock due to insufficient ventilation and when agitation of manure during storgage emptying occurs (Ni et al., 2010). At high concentrations hydrogen sulfide is an eye and respiratory tract irritant in humans (Cole et al., 2000). Usually the concentration in closed swine facilities (<10 ppm) is lower than the concentration of other gases like ammonia and carbon dioxide (Holland et al., 2002; Jacobson et al., 2003). From 250 ppm onwards, pigs can experience distress (O'Donoghue, 1961). Hydrogen sulphide can irritate the mucous membranes of the eye and respiratory tract and in the deeper airways it can result in pulmonary oedema (Curtis, 1983). Concentrations of $H_2S$ between 500 and 1000 ppm or higher, which may occur when manure pits are agitated, can cause death or intermittent spasms, cyanosis, unconsciousness and convulsions in both human and pigs (Donham et al., 2000; Ensley and Osweiler, 2012; Holland et al., 2002; Lillie, 1972; O'Donoghue, 1961; Stombaug et al., 1969).

Carbon dioxide is produced by the animals and to a lesser extent originates from the manure (Ni et al., 1999; Philippe and Nicks, 2015). Measuring $CO_2$ concentrations can be used to evaluate the ventilation efficiency. Indoor air $CO_2$ concentrations were between 350 and 4350 ppm in mechanically ventilated pig houses (Anderson et al., 1987; Jacobson et al., 1996) and between 1430 and 3050 ppm in naturally ventilated pig houses (Jacobson et al., 1996; Van't Klooster and Heitlager, 1994). The production of respiratory $CO_2$ corresponds to 2.23, 3.68, 0.88 and 1.70 kg $CO_2$ $head^{-1}$ $day^{-1}$ for gestating sows, lactating sows, weaned piglets and fattening pigs, respectively (CIGR, 2002). The maximum allowed concentration for animals is 3000 ppm (CIGR, 1992). After 6 to 8 h failing of the ventilation system, asphyxiation of animals inside the room may occur by displacement of oxygen by $CO_2$ (Donham et al., 1982; Ensley and Osweiler, 2012). Not much attention is paid to $CO_2$ when evaluating the air quality in relation to respiratory health of the pig, however one report found that sneezing, coughing or pneumonia is higher in pigs experiencing $CO_2$ concentrations of 2000-9000 ppm compared to 1000-3000 ppm (Busse, 1993; Ni et al., 2000).

Methane originates from the anaerobic degradation of organic matter performed by bacteria in the digestive tract of the pigs and in the manure (Philippe and Nicks, 2015). Less information of the impact of this gas on pig health is available, as it is considered to be a significant contributor to greenhouse gas formation and not as important as compromiser of pig health (Ni et al., 1999). Not much information of the impact of $CH_4$ in terms of impact on animal health is available. It is reported that concentrations of 50 000 ppm are hazardous for the animal (Muehling, 1970; Ni et al., 1999), but rather as explosion danger, as this concentration is the lower explosion limit of $CH_4$ (Yaws, 2001). Mostly concentrations will stay below this level (Hartung and Phillips, 1994).

A summary on the recommendations on maximum gas concentrations is given in table 3.

**Table 3: Recommendations on maximum gas concentrations in pig stables**

| Gas | Maximum concentration (ppm) |
|---|---|
| $NH_3$ | 20 |
| $H_2S$ | 0.1-0.2<br>0.5* |
| $CO_2$ | 3000 |
| $CH_4$ | NA |

Adapted from Ni, 1998 (Ni, 1998): *Intermittently, when dunging out, $NH_3$: ammonia, $H_2S$: hydrogen sulphide, $CO_2$: carbon dioxide, $CH_4$: methane, NA: not available

### 1.6.4.3. Ventilation, air velocity and relative humidity

Ventilation has a direct impact on aerosol levels and has been found to be associated with respiratory disease levels (Stärk, 2000). An air exchange of >60 $m^3$ per hour per pig has a protective effect on pneumonia (Flesjå et al., 1982). Special attention should be paid to temperature set points, fan staging, air inlet, curtain settings, sensor placement, heating capacity, preventing draughts and building maintenance when evaluating housing systems in order to reduce respiratory disease (Gonyou et al., 2006; Heinonen, 2001; Maes et al., 2008; Mul and Vermeer, 2010). Improving ventilation strategies (forced vs. natural), will decrease the prevalence of *M. hyopneumoniae*-infections (Fraile et al., 2010). Air velocity is inherently connected to the thermal comfort of the pig. The air velocity at the pig level should be maximum 0.2 m/s (see further) (D. Maes, personal communication). A limited number of studies determine the influence of cold air draughts on the immune response in pigs. Sheepens et al. (1994) concluded that climatic stress disturbs the homeostasis of the pigs' immune system and increases the activity level of the pigs, which increases the particulates levels and the risk of transmitting respiratory agents via aerosols. Pathogens are best controlled under the circumstances of 60-80% relative humidity (Gonyou et al., 2006). Alterations of the air humidity have adversely affect the viability of microorganisms. Bacterial cells tend to die as they dry, but once dry, they can remain viable for long periods, if kept in a dry state. Survival of bacterial cells is lowest at midrange relative humidity. This is also true for *Mycoplasma* species. The effect of the relative humidity on the survival of airborne viruses varies with the type of microorganism (Harry, 1978). It was observed that a large variation in indoor humidity was also significantly associated with the prevalence of pneumonia, but the mechanism for this association is not clear (Done, 1991).

### 1.6.4.4. Thermal environment

Temperature can be considered as a factor of climatic stress. Quantitative data between the association of temperature with respiratory health is limited in pigs (Stärk, 2000). Cold temperatures have an inhibitory capacity to clear bacteria from the lungs, probably due to a decreased mucociliary clearance induced by cold temperatures, but this effect becomes less important as the pigs become older (Curtis et al., 1976; Giesbrecht, 1995). The

outside environmental condition strongly influences the inside conditions in fattening units, as in most fattening units no additional heating is provided (Stärk, 2000). The lower the outside temperature, the higher the probability of pigs being seropositive for *M. hyopneumoniae* (Segalés et al., 2012). Conversely, high outdoor temperatures are associated with increased pneumonia as well (Done, 1991). Not only the temperature itself is important, but temperature fluctuations also need to be avoided (Gonyou et al., 2006). Fattening pigs can tolerate temperature fluctuations of 5-10 °C within 24 hours at an air velocity of 0.15 m/s (D. Maes, personal communication). A 12°C or more variation in temperature within 24 hours increases the incidence of pneumonia (Done, 1991). It was shown that fluctuating temperatures (from 4°C to 30 °C) enhanced the progress and severity of Aujeszky's disease in pigs (Narita et al., 1992) and the immune response of pigs challenged with *A. pleuropneumoniae* was significantly influenced by temperature, together with draughts (Kreukniet et al., 1990; Noyes et al., 1986).

Goodwin (1985) found a tendency for EP to start especially in autumn and winter and this was confirmed by Jorsal and Thomson (1988). On the other hand, Stärk et al. (1992) found that onset of clinical disease started in November-March, under European conditions. Segalés et al. (2012) described that animals born in autumn (when higher rainfall occurred), entering the finishing units in winter (when lower temperatures were recorded in that study) and reaching the slaughter age in spring, had higher probabilities to be infected with *M. hyponeumoniae* and to be seropositive to *M. hyopneumoniae*. Their explanation was that in Spain the winters are cold and wet and in summer the climate is hot and dry, favoring *M. hyopneumoniae* survival in their winters (humid, low temperature and lower amount of UV). Logically, this cannot be generalized to other countries/regions with different climate conditions. Zhuang et al (2002) concluded that rainfall and especially temperature were negatively associated with the seasonal variation in occurrence of *M. hypopneumoniae*-infections. This was confirmed by Vangroenweghe et al. (2016) who found a negative association of rainfall in the week preceding sampling of three to five week old pigs with the probability of these pigs testing positive for *M. hyopneumoniae* on tracheobronchial swabs.

### 1.6.5. Influence of other infectious agents and mycotoxins

Single infection with *M. hyopneumoniae*, although uncommon under commercial circumstances, is considered to result in mild clinical signs (Sibila et al., 2009). In uncomplicated cases, the pigs can be (subclinically) infected with no evidence of coughing and pulmonary lesions (Fano et al., 2005). In the case of secondary bacterial infection, clinical signs will be more pronounced such as fever, laboured breathing, prostration and reduced appetite. This causes an increased feed conversion ratio, lower daily growth and uneven growth (Del Pozo Sacristán, 2014). Next to bacterial agents, also viral pathogens can play a role in the development of respiratory disease in pigs. Combined with many non-infectious factors discussed before, this complex is referred to as Porcine Respiratory Disease Complex (PRDC). Primary pathogens in this complex, next to *M. hyopneumoniae* are PRRSv, Porcine Circovirus type 2 (PCV2), swine influenza virus (SIV), *Actinobacillus pleuropneumoniae (A. pleuropneumoniae)*, *Bordetella bronchiseptica (B. bronchiseptica)* and Aujeszky's disease virus in countries not free of the pathogen. Other secondary pathogens are: *Trueperella pyogenes*, *Streptococcus suis* (*S. suis)*, *P. multocida* , *M. hyorhinis*, *H. parasuis*, *Actinobacillus suis*, Porcine Respiratory Coronavirus, Torque teno sus virus, Porcine Cytomegalovirus and Paramyxovirus (Opriessnig et al., 2011). In most cases the interaction of *M. hyopneumoniae* and other pathogens

potentiates the effect. An overview of interactions in polymicrobial respiratory diseases is given by Opriessnig et al. (2011).

Mycotoxins are secondary fungal metabolites which can contaminate many types of food crops (Vandenbroucke, 2012). *Fusarium* mycotoxins are the largest group of mycotoxins, which includes more than 140 known metabolites of fungi (Sobrova et al., 2010). *Fusarium* species are the most prevalent mycotoxin producing fungi in Europe. This may be due to the moderate climate conditions in Europe, as they require somewhat lower temperatures for growth and mycotoxin production than, for instance *Aspergillus* species (Placinta et al., 1999). Trichothecenes, zearalenone, fumonisin and moniliformin are the mycotoxins produced by *Fusarium* fungi which are most important in terms of contamination of feed crops intended for food-producing animals (Sobrova et al., 2010; Vandenbroucke, 2012). The trichothecenes causing most concern are T-2 toxin, which is the most acute toxic trichothecene, HT-2 toxin, nivalenol and deoxynivalenol (DON) (Eriksen and Pettersson, 2004). Deoxynivalenol is the predominant mycotoxin of the trichothecenes group and is mainly produced by *F. graminearum* and *F. culmorum* (Antonissen et al., 2014b; McMullen et al., 1997). Deoxynivalenol is also known as vomitoxin, due to the strong emetic effects after consumption by influencing the dopaminergic receptors in the brain (Sobrova et al., 2010) and is typically found in wheat, corn, barley and rye (Broeckaert et al., 2015). In twelve European countries, mycotoxin testing on 11,022 food samples resulted in DON being the most prevalent mycotoxin with a result of 57 percent of all samples being positive for this mycotoxin (European Food Safety Authority, 2013). In another three-year survey of Rodrigues and Naehrer (2012) 59% of 5,819 animal feed samples tested positive for the presence of DON and the average contamination level was 1,104 μg/kg, whereas the maximum observed level was 49,307 μg/kg. As described by Monbaliu et al. (2010), 67 of 82 feed matrices (sow feed, wheat and maize) were contaminated with mycotoxins, of which 52 were contaminated with DON. Swine are very sensitive to the effects of DON, mainly because of differences in metabolism of this mycotoxin compared to other animal species (Bracarense et al., 2012; Wu et al., 2010) and the high cereal content of their diets (Pierron et al., 2016). The maximum recommended level set by the European Commission for DON in products intended for pig feeding is 0.9 mg/kg which is the lowest value compared to other species (table 4) (Commission recommendation of 17 August, 2006).

Van Asselt et al. (2012) demonstrated that acetylated forms of DON, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), are more prevalent than DON itself. Their effect was unrecognised, therefore not much attention was paid to these metabolites in mycotoxin feed analyses, until Broeckaert et al (2015) demonstrated that the pig is able to hydrolyse 100% of the acetyl-derivates 3-ADON and 15-ADON ingested *per os* to DON.

Eriksen and Pettersson (2004) stated that changes in biochemical parameters (such as plasma nutrients and plasma enzymes) are possible, but this effect is partly caused by the reduced feed intake and is not a direct effect of the toxin. Effects of moderate to high levels of DON are well known and include decreased feed intake, feed refusal, decrease in weight gain, effects on intestinal health, dysfunction in spleen and liver, lower oocyte and slower embryo development, alteration and delay of the immune response (cellular and humoral) and increased disease susceptibility (Antonissen et al., 2014a; D'Mello et al., 1999; Dersjant-Li et al., 2003; Forsyth et al., 1977; Gerez et al., 2015; Goyarts and Dänicke, 2006; Swamy et al., 2003; Tiemann and Dänicke, 2007). Very high doses

of DON, even though these concentrations are not likely to be encountered in animal feed, result in abdominal distress, malaise, diarrhoea, emesis and even shock or death, (Antonissen et al., 2014a; Bracarense et al., 2012; Cheat et al., 2015). In literature, however it is not mentioned from which concentration onwards these symptoms are observed. It can be expected that the effects of mycotoxin on intestinal health and immune responses, may also influence the susceptibility to infections with pathogens (Antonissen et al., 2015; Antonissen et al., 2014a). In fact, DON has a known impact on the pathogenesis of several bacterial or viral diseases. Pig ileal loop tissues exposed to a DON-concentration of 1 μg/ml, were significantly more invaded by *Salmonella* Typhimurium and showed subsequently increased inflammatory responses compared to control loops (Vandenbroucke et al., 2011). Furthermore, DON in a concentration of 0.025 μg/ml, was able to induce an enhanced uptake of *Salmonella* Typhimurium in porcine macrophages, indicating the capacity of DON to alter the susceptibility of the pig to *Salmonella* Typhimurium infections by modulating the innate immune system. The impact of *Fusarium* mycotoxins on respiratory infections with bacterial or viral agents in the pig is summarized in table 5.

**Table 4: The maximum recommended level on the presence of deoxynivalenol and fumonisin B1+B2 in products intended for animal feeding, as determined by the European Commission (Adapted from Recommendation of 17 August 2006 (Commission recommendation of 17 August, 2006)**

| Mycotoxin | Products intended for animal feed | Maximum level (ppm) |
|---|---|---|
| Deoxynivalenol | Feed materials (*) | |
| | — Cereals and cereal products (**) with the exception of maize by-products | 8 |
| | — Maize by-products | 12 |
| | Complementary and complete feedingstuffs with the exception of: | 5 |
| | — complementary and complete feedingstuffs for pigs | 0.9 |
| | — complementary and complete feedingstuffs for calves (< 4 months), lambs and kids | 2 |
| Fumonisin B1 + B2 | Feed materials (*) | |
| | — maize and maize products(***) | 60 |
| | Complementary and complete feedingstuffs for: | |
| | — pigs, horses (Equidae), rabbits and pet animals | 5 |
| | — fish | 10 |
| | — poultry, calves (< 4 months), lambs and kids | 20 |
| | — adult ruminants (> 4 months) and mink | 50 |

(*) that the use of cereals and cereal product and their use in a daily ration should not lead to the animal being exposed to a higher level of these mycotoxins than the corresponding levels of exposure where only the complete feedingstuffs are used in a daily ration.

(**) The term 'Cereals and cereal products' includes feed materials referred to in part B of the Annex to Council Directive 96/25/EC of 29 April 1996 but also other feed materials derived from cereals in particular cereal forages and roughages.

(***) The term 'Maize and maize products' includes not only the feed materials derived from maize listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in the Annex, part B of Directive 96/25/EC but also other feed materials derived from maize in particular maize forages and roughages.

**Table 5 Effect of *Fusarium* mycotoxins on respiratory infections in pigs**

| Fusarium mycotoxin | Dose | Age of the pig (weight) | Exposure period | Pathogen | Effect[a] | Type of study | Reference |
|---|---|---|---|---|---|---|---|
| FB1, FB2, FB3 | FB1: 20 mg/kg feed, FB2: 3.5 mg/kg feed, FB3: 1.9 mg/k feed | 3 d (2.35±0.20kg) | 42 d | *M. hyopneumoniae* | dyspnea and hypoxia in 1/7 pigs of the *M. hyopneumoniae* and FB-infected group<br>No effect of FB in av. pulmonary pixel density on CT-scan in *M. hyopneumoniae*-infected pigs | *in vivo* | (Pósa et al., 2013) |
| FB1 | 10 mg/kg feed | 3 d (not mentioned) | 24 d | *B. bronchiseptica*<br>*P. multocida* (serotype D) | Clinical signs (sneezing, serous nasal discharge, panting, hoarseness) and lung lesions in 4/7 pigs in infected group and 6/7 in infected + fumonisin B1 group | *in vivo* | (Pósa et al., 2011) |
| FB1 | 0.5 mg/kg BW | Piglets, age not mentioned (9.6±2.1 kg) | 7 d | *P. multocida* (serotype A) | ↓ ADG, ↑ coughing rate, ↑ MLL and histopathological lesions of the lung, ↑ # of MØ and lymphocytes in BALF in FB1+ *P. multocida* group | *in vivo* | (Halloy et al., 2005) |
| FB1 | 12 mg/kg BW | 29 d (5.8 kg) | 18 d | PRRSv | ↑ histopathological lesions in the group PRRSv inoculated at D8 and administered FB1 | *in vivo* | (Ramos et al., 2010) |
| DON | 0, 70, 140, 280, 560, 1200 ng/ml | N.A. | 72 h | PRRSv | ↑ survival of non-PRRSv-infected MARC-145 cells and PAM's in a dose dependent manner<br>140 and 280 ng/ml DON increased survival of PRRSv-infected cells by ↓ PRRSv replication | *in vitro* | (Savard et al., 2014b) |
| DON | 0, 70, 140, 280, 560, 1200 ng/ml | N.A | 72 h | PCV2a<br>PCVb | ↑ PCV2b replication in NPTr-cells in the concentration range of 70-280 ng/ml | *in vitro* | (Savard et al., 2015) |
| DON | 0, 2.5, 3.5 mg/kg feed | 21 d | 21 d | PCV2b | Viremia and lung viral load tended to be ↑ in 2.5 mg/kg feed group<br>No impact of DON on clinical manifestation of PCVAD | *in vivo* | (Savard et al., 2015) |
| DON | 0, 2.5, 3.5 mg/kg feed | 21 d | 28 d | PRRSv | Significant effect of 3.5 mg/kg DON on ADG, MLL, mortality<br>Negative effect of 2.5 mg/kg DON on PRRSv specific Ab concentrations in serum | *in vivo* | (Savard et al., 2014a) |

[a]Effect of mycotoxin exposure is compared to the negative control, unless stated otherwise. Adapted from (Antonissen et al., 2014a).

FB1: fumonisin B1, FB2: fumonisin B2, FB3: fumonisin B3, d: days: *M. hyopneumoniae*: *Mycoplasma hyopneumoniae*, av.: average, CT: computed tomography, *B. bronchiseptica*: *Bordetella bronchiseptica*, *P. multocida*: *Pasteurella multocida*, BW: body weight, ADG: average daily gain, MLL: macroscopic lung lesions, #: number, MØ: macrophages, BALF: broncho-alveolar lavage fluid, PRRSv: Porcine Reproductive and Respiratory Syndrome virus, D: day of the study, N.A.: not applicable, h: hours, PAM: porcine alveolar macrophages, PCV2: porcine circo virus type 2, NPTr: newborn pig trachea epithelial cell line, PCVAD: porcine circovirus associated disease, Ab: antibodies

### 1.6.6. Vaccination

Vaccination against *M. hyopneumoniae* is applied worldwide: as many as 70% of pig herds are vaccinating against *M. hyopneumoniae* (Maes et al., 2008; Martelli et al., 2014). Vaccination against *M. hyopneumoniae* does not protect the pigs from being colonized, nor offers a complete protection against clinical signs and appearance of lung lesions (Mateusen et al., 2001; Mateusen et al., 2002; Meyns et al., 2004). Vaccination against *M. hyopneumoniae* has been proven to reduce clinical signs, pneumonia lesions and economic losses associated with the infection, such as improving daily weight gain (2-8%), feed conversion ratio (2-5%), time to reach slaughter weight and occasionally a lower mortality and an improved carcass quality (Jensen et al., 2002; Maes et al., 1998; Maes et al., 1999; Muirhead and Alexander, 1997; Ross, 1999). Additionally, the use of vaccination may reduce the number of *M. hyopneumoniae* organisms in the respiratory tract and may decrease the infection level in a herd (Meyns et al., 2006; Thacker et al., 1998). These beneficial effects should be expected several months after initiating a *M. hyopneumoniae* vaccination program and should always be accompanied, if not initiated with other control measures (Haesebrouck et al., 2004; Maes et al., 2008). The mechanism of protection against *M. hyopneumoniae* through vaccination is still unclear (Desrosiers, 2001). Vaccination against *M. hyopneumoniae* induces local, mucosal, humoral and cellular immunity. It has been suggested that mucosal antibodies, modification of the inflammatory response and cell-mediated immune responses are important vaccination-responsive factors in controlling the disease (Thacker et al., 2000a).

Criteria to vaccinate against *M. hyopneumoniae* have been suggested by Muirhead and Alexander (1997): presence of *M. hyopneumoniae* in the herd, primary or secondary respiratory infections, variable or poor growth associated with respiratory disease in the herd, weaning to slaughter mortalities >4%, the necessity to apply in-feed medication and the cost of vaccination ≤ cost of mortality and in-feed medication. Currently, the prudent use of antimicrobials to combat resistance might as well be one of the decisions to vaccinate against *M. hyopneumoniae*. All commercially available vaccines are bacterins, and therefore mainly consist of killed *M. hyopneumoniae* bacteria. Many commercially available *M. hyopneumoniae* vaccines are constituted of the same strain, the J-strain but are formulated with a different adjuvant. The water based adjuvants, like aluminium salts are relatively mild and stimulate the immune system to a lesser extent. They are used for a prime-boost regime. Compared to the water based adjuvants, the oil-based adjuvants, show a prolonged release of the antigen and stimulation of the immune response, mimicking the two-dose prime-boost regime vaccines, but are administered only once (Del Pozo Sacristán, 2014; Yuki and Kiyono, 2003). The type of adjuvant is important since it steers the induced immune response towards a certain direction and therefore may influence the level of protection. An overview of the commercially available vaccines against *M. hyopneumoniae* is given by Maes et al. (2017).

Deciding which vaccination strategy should be implemented needs to be based on the type of herd, production system, and the infection pattern in the herd (Haesebrouck et al., 2004). In general, the main strategy of *M. hyopneumoniae*-vaccination is directed towards vaccinating the piglets before or at weaning, while sow vaccination is rarely practiced (Bargen, 2004; Del Pozo Sacristán, 2014; Sibila et al., 2008).

Vaccination of gilts before entering the sow herd, may be advised to avoid destabilisation of the herd immunity. Especially when gilts are purchased from *M. hyopneumoniae*-free farms, it is strongly advised to vaccinate these gilts (Bargen, 2004).

Sow vaccination is based on the strategy to passively immunize their offspring via colostral antibodies and to decrease (though not prevent) vertical transmission via nose-to nose contact from sow to piglet (Ruiz et al., 2003; Sibila et al., 2008; Thacker et al., 2000a). It was demonstrated that sow vaccination did not have an impact on colonisation of *M. hyopneumoniae* in the piglets, but the severity of pneumonia in those piglets was reduced (Sibila et al., 2008). The effect from vaccinating the gilts and sows against *M. hyopneumoniae* on the efficacy of piglet vaccination against *M. hyopneumoniae* remains debated. The effect on the efficacy of vaccination in the piglets of this interference is unclear as post-vaccination serum antibody levels have limited utility in determining the degree of protection afforded by vaccination (Haesebrouck et al., 2004). Regardless, vaccination against *M. hyopneumoniae* in Europe is performed in the presence of maternal antibodies, with good results (Maes et al., 2008; Martelli et al., 2006). Other authors report interference with building up an active immunity when vaccinating piglets in face of maternal antibody titers in the piglets (Jayappa et al., 2001). Bandrick et al. (2014) found that piglets with *M. hyopneumoniae* specific maternally derived immunity indeed failed to show a rise in vaccine induced *M. hyopneumoniae* antibody levels, but did show primary (antigen-specific lymphoproliferation) and secondary (delayed type hypersensitivity) *M. hyopneumoniae*-specific cell-mediated immunity responses following vaccination.

Vaccination of suckling piglets (early vaccination, < 4 weeks of age) is frequently applied (Del Pozo Sacristán, 2014), as in this case, active immunity is established before the piglets are exposed to the pathogen (Del Pozo Sacristán, 2014; Maes et al., 2008). This vaccination strategy is frequently applied in single-site herds.

Late vaccination (between 4 and 10 weeks of age) may be applied in herds where the infection occurs during the finishing period, mostly in multi-site systems (Haesebrouck et al., 2004; Maes et al., 2008), in herds with strict all-in/all-out production, or when segregated early weaning is practised. It is not recommended in herds with continuous pig flow and in herds where the pigs are infected early in life (Haesebrouck et al., 2004). Vaccinating nursery piglets has the advantage that the possible interference with maternal immunity is lower. However, nursery pigs might already be infected with *M. hyopneumoniae* and other pathogens such as PRRSv and PCV2, which might interfere with building up a proper immune response and might therefore not be the perfect moment for vaccination (Campbell et al., 2013; Maes et al., 2008; Pié et al., 2004).

Single-dose vaccines applied at 3 weeks of age or older confer similar effects as double vaccination (Baccaro et al., 2006; Haesebrouck et al., 2004). Single-dose vaccines reduce labour, cause less stress for the pigs and can easily be implemented in routine management practices in the farm (Del Pozo Sacristán et al., 2014). In some cases, double-dose vaccination for *M. hyopneumoniae* are still advised: multi-source/age purchase policy, continuous flow of the pigs instead of AI/AO policy, during critical periods (pigs placed in the fattening units in fall because of added environmental stress) and large herds with unstable PRRSv or swine influenza virus situation (Yeske, 2001).

Although it is clear that vaccinating against *M. hyopneumoniae* has beneficial effects in most herds, these effects can vary between herds (Maes et al., 2008). *Mycoplasma hyopneumoniae* vaccine efficacy can be reduced by incorrect injection technique and improper storage of the vaccine. Also infection with other pathogens, such as *Ascaris*

*suum* (Steenhard et al., 2009) or PRRSv (Thacker et al., 2000b) during or shortly after vaccination has shown to decrease the response of vaccinating against *M. hyopneumoniae*. Another factor to consider is the antigenic difference between vaccine strain and circulating field strains (Maes et al., 2008).

Intramuscular injection is the main route of administration of *M. hyopneumoniae* vaccination. Intradermal vaccination against *M. hyopneumoniae* can be an asset, as specialised antigen presenting cells are necessary to obtain a good cellular immune response and more of these specialised cells are present in the skin compared to the muscle (Bos and Kapsenberg, 1986; Fu et al., 1997; Iwasaki et al., 1997; Martelli et al., 2009). In addition, no needles are used but the vaccine is administered intradermal via pressure. This may reduce the risk for iatrogenic infections and the higher dispersion of the antigen at the site of injection reduces injection site reactions (Del Pozo Sacristán, 2014). Intradermal vaccination is already being applied for *M. hyopneumoniae* with good results (Ferrari et al., 2011; Jones et al., 2005; Martelli et al., 2009; Martelli et al., 2014; Tassis et al., 2012). Beffort et al. (2017) compared the safety and efficacy of intradermal versus intramuscular vaccination against *M. hyopneumoniae* and observed less injection site reactions and a superior reduction of prevalence and severity of lung lesions in the intradermal vaccinated group. Other administration routes like aerosol vaccination (Murphy et al., 1993), oral vaccination (Lin et al., 2003; Ogawa et al., 2009), intranasal vaccination (Shimoji et al., 2003) and intraperitoneal vaccination (Sheldrake et al., 1991) have been investigated, with variable results. These administration routes are currently not used in practice.

Combination vaccines contain antigens from different pathogens in the same vaccine formulation. The current objectives in modern pig husbandry necessitate maximising vaccine benefits and minimising cost, labour for the farmer and stress for the pig (Drexler et al., 2010). Studies that evaluate the effect of different combination vaccines are available: Drexler et al. (2010), Bourry et al. (2015) and Jiménez and Lopéz (2007) studied the effect of combining *M. hyopneumoniae* and PRRSv vaccination, Witvliet et al. (2015); Park et al. (2016), Jeong et al., (2016) and Hayes and Saltzman (2009) investigated the combined effect of *M. hyopneumoniae* and PCV2 vaccination, while Delisle and Rigaut (2007) investigated the combined effect of PRRSv, *M. hyopneumoniae* and *A. pleuropneumoniae* vaccination. These authors showed that the above mentioned vaccines may be combined without compromising the efficacy and safety. However, the instructions in the product leaflet should be followed strictly and only vaccines that are registered and therefore thoroughly tested should be used in combination vaccines.

There is a need to produce more effective and lower cost vaccines against EP. Questions regarding the efficacy of the existing vaccines remain, and cultivating *M. hyopneumoniae* to produce bacterins is expensive (Kobish and Friis, 1996; Simionatto et al., 2013). In addition, the slow growth rate and fastidious nutrient requirements make it very challenging to reach sufficient biomass for antigen production (Kamminga et al., 2017). Several studies have used recombinant DNA technology approaches to find new vaccine candidates and to investigate their suitability (Marchioro et al., 2014; Marchioro et al., 2012; Simionatto et al., 2013). An overview of experimental vaccines is provided by Simionatto et al. (2013) and Maes et al. (2017). The availability of complete genome sequences gave a boost to the reverse vaccinology approach. This approach starts from genome sequencing results and subsequently predicts antigens by 'in silico' analysis as promising candidates for vaccines (Rappuoli, 2001). The combination of recombinant DNA technology and 'in silico' analysis of whole genome sequences has the advantage that the pathogen is being excluded from the vaccine production process (Liljeqvist and Stahl,

1999; Movahedi and Hampson, 2008). The disadvantage is that it is difficult to correlate these antigens with protection and that the reverse vaccinology approach for antigens like glycolipids and lipopolysaccharides, which are important components of vaccines, cannot be employed (Rappuoli, 2001; Serruto and Rappuoli, 2006). Further research is being carried out to develop more effective vaccine candidates against *M. hyopneumoniae*.

### 1.6.7. Antimicrobial medication

Antimicrobials can help controlling EP, but are not able to eliminate the organism from the respiratory tract, nor heal existing lesions. For the success of the therapy, it is important to know the secondary pathogens present in the lower respiratory tract and their antimicrobial susceptibility profile (Thacker and Minion, 2012). Lack of this knowledge might be one of the reasons why treatment of EP is not always successful (Desrosiers, 2001; Ross, 1999).

Antimicrobials applied as individual treatments (parenteral administration) are favoured in the acute phase of respiratory disease when pigs have a reduced appetite or refuse to drink (Henry and Apley, 1999; Pijpers et al., 1990). Group treatments via in-feed or in-water medication are less labour intensive and cause less stress compared to parenteral treatment (Del Pozo Sacristán, 2014). Antimicrobials can be used in a therapeutic, metaphylactic or prophylactic way. Therapeutic treatment involves episodic use of therapeutic doses of antimicrobial agents for management of specified infectious diseases in clinically ill animals (Paulson et al., 2015). Metaphylactic use is defined as treating the whole group when some pigs are clinically diseased and others subclinically infected. Prophylactic or preventive use is medicating a group of pigs during high-risk periods to prevent them from a possible clinical outbreak (Murphy et al., 2017; Vicca et al., 2005).

All *Mycoplasma* species are resistant against beta-lactam antimicrobials (such as penicillin, ampicillin, amoxicillin and cephalosporins), because they lack a cell wall, which is the target of these antimicrobials. Classes of antimicrobials working against *M. hyopneumoniae* are tetracyclines, macrolides, lincosamides, pleuromutilins, amphenicols, aminoglycosides, aminocyclitols and fluoroquinolones (Holst et al., 2015). An excellent overview of the mode of action and the spectrum of activity of these antimicrobials against *M. hyopneumoniae* is provided by Del Pozo Sacristán et al. (2014). Because of the importance of fluoroquinolones in human medicine, the use of these antimicrobials should be avoided (Desrosiers, 2001; WHO, 2012), unless susceptibility tests show that *M. hyopneumoniae* is resistant to other antimicrobials, except fluoroquinolones. In practice susceptibility testing is not feasible, as *M. hyopneumoniae* is difficult to culture, hence antimicrobial sensitivity tests for *M. hyopneumoniae* are not performed routinely (Friis, 1975; Thacker and Minion, 2012). Acquired resistance against tetracyclines, macrolides, lincosamides and fluoroquinolones has been documented (Hannan et al., 1997; Inamoto et al., 1994; Vicca et al., 2007; Vicca et al., 2004).

A decade ago, the choice between implementing vaccination or administration of antimicrobials was debated: antimicrobials are flexible in use, several (respiratory) pathogens are addressed, if in-feed or in-water medication is used, they are less labour intensive to be applied compared to vaccinating and they have an immediate effect, while the effect of vaccination will be noticeable after some months (Del Pozo Sacristán, 2014; Haesebrouck et al., 2004; Maes et al., 2008). Mateusen et al. (2001) showed that the use of tilmicosin compared to implementing vaccination against *M. hyopneumoniae* conferred similar beneficial effects. Combination vaccines, nowadays can be

applied for up to two agents (viral and bacterial agents). Antibiotic use increases the risk for residues in the carcass at slaughter and select for antimicrobial resistance in commensal, pathogenic and/or zoonotic bacteria in the animal (Maes et al., 2008).

Prudent use of antimicrobials to combat antimicrobial resistance is a priority for the European Commission. At the EU level, the monitoring and reporting of antimicrobial resistance in bacteria associated with the main livestock animal species (cattle, pigs, poultry) and derived food is regulated by Commission Implementing Decision 2013/652/EU (Murphy et al., 2017). Different countries have implemented national programs to monitor and reduce the use of antimicrobials in livestock (Callens et al., 2012). In fact, AMCRA (Antimicrobial Consumption and Resistance in Animals) advises no first choice antimicrobials for the treatment of EP, but suggests that preventive measures should be implemented, preventive treatments are strongly discouraged (AMCRA, 2018). On the other hand, even in herds vaccinating against *M. hyopneumoniae*, clinical disease can remain (Mateusen et al., 2002) and the use of antimicrobials may therefore be necessary to control respiratory disease (Callens et al., 2012; Del Pozo Sacristán, 2014; Maes et al., 2008; Mateu and Martin, 2001; McEwen and Fedorka-Cray, 2002; Schwarz et al., 2001; Timmerman et al., 2006). Additionally, eradication programs for *M. hyopneumoniae* based on partial sanitation often require the use of antimicrobials. Eradication programs based on total sanitation, where all animals from the farm are sold or culled and the farm is populated with *M. hyopneumoniae*-free pigs, does not include the use of antimicrobial agents (Holst et al., 2015; Maes et al., 2008; Zimmerman et al., 1989). A recent overview of current eradication protocols, including the protocols with or without the use of antimicrobials is provided in Holst et al., (2015).

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## CHAPTER 2: AIMS OF THE STUDY

# *Aims of the Study*

# AIMS OF THE STUDY

*M. hyopneumoniae* is the primary pathogen of enzootic pneumonia, and one of the most important pathogens involved in the porcine respiratory disease complex. Infections with *M. hyopneumoniae* negatively impact growth and feed efficiency, increase antimicrobial use and facilitate infections with other respiratory pathogens.

The outcome of *M. hyopneumoniae* infections is quite variable, ranging from subclinical infection to severe clinical disease leading to tremendous economic losses for the pig sector. It has been shown that many different factors such as the virulence of *M. hyopneumoniae* strain, environmental and management factors and vaccination strategies may influence the severity of infection. However, the impact of many factors such as the diversity of *M. hyopneumoniae* strains, the concentration of air pollutants in the stable, and the presence of mycotoxins in the feed is still not yet fully understood. In addition, the currently available vaccines are mostly based on the J-strain which was isolated in the second half of the previous century. These vaccines are widely used and are cost-efficient in many herds. Although they provide partial protection against clinical disease and lung lesions, they do not prevent infection.

The general aim of this thesis was to investigate factors that influence the outcome and the severity of *M. hyopneumoniae* infections in order to achieve a better control of the disease.

The specific objectives were:

1. To determine the diversity of *M. hyopneumoniae* in Flemish pig farms and to investigate its impact on lung lesions at slaughter
2. To investigate the impact of particulate matter and ammonia levels in the stable on pneumonia and pleurisy lesions
3. To assess the impact of deoxynivalenol and acetyl-derivates in the feed on an experimental *M. hyopneumoniae* infection
4. To investigate the effect of vaccination with a commercial vaccine on an experimental *M. hyopneumoniae* challenge-infection with two genetically different *M. hyopneumoniae* strains

# CHAPTER 3: FIELD AND EXPERIMENTAL STUDIES

# *Field and experimental studies*

# *3.1. Impact of diversity of Mycoplasma hyopneumoniae strains on lung lesions in slaughter pigs*

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## Abstract

The importance of diversity of *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) strains is not yet fully known. This study investigated the genetic diversity of *M. hyopneumoniae* strains in ten pig herds, and assessed associations between the presence of different strains of *M. hyopneumoniae* and lung lesions at slaughter. Within each herd, three batches of slaughter pigs were investigated. At slaughter, from each batch, 20 post mortem bronchoalveolar lavage fluid samples were collected for multiple locus variable-number tandem repeat analysis (MLVA), and lung lesions (*Mycoplasma*-like lesions, fissures) were recorded. Multivariable analyses including potential risk factors for respiratory disease were performed to assess associations between the number of different strains per batch (three categories: one strain, two-six strains, ≥ seven strains), and the lung lesions as outcome variables. In total, 135 different *M. hyopneumoniae* strains were found. The mean (min.-max.) number of different strains per batch were 7 (1-13). Batches with two-six strains or more than six strains had more severe *Mycoplasma*-like lesions (P= 0.064 and P= 0.012, respectively), a higher prevalence of pneumonia (odds ratio (OR): 1.30, P=0.33 and OR: 2.08, P=0.012, respectively), and fissures (OR= 1.35, P=0.094 and OR= 1.70, P=0.007, respectively) compared to batches with only one strain. In conclusion, many different *M. hyopneumoniae* strains were found, and batches of slaughter pigs with different *M. hyopneumoniae* strains had a higher prevalence and severity of *Mycoplasma*-like lung lesions at slaughter, implying that reducing the number of different strains may lead to less lung lesions at slaughter and better respiratory health of the pigs.

## Introduction

*Mycoplasma hyopneumoniae (M. hyopneumoniae)* is the causative agent of enzootic pneumonia, and infections occur in all countries with an intensive pig production (Thacker, 2004). Infections with *M. hyopneumoniae* cause tremendous economic losses, either directly or indirectly, by increasing the susceptibility of infected animals to other respiratory pathogens (Thacker, 2006).

*Mycoplasmas* have small genomes (580 to 1,300 kb) (Hutchison and Montague, 2002; Minion et al., 2004), and genetic diversity might be one solution to adapt to the adverse environment of the host (Madsen et al., 2007; Vranckx et al., 2012). Many regions in the genome of *M. hyopneumoniae* related to adherence in the host contain variable number of tandem repeats (VNTRs). These regions are prone to recombination events and slipped strand mispairing, which can possibly lead to expression of a different sized protein (Torres-Cruz and van der Woude, 2003). Multiple locus variable number of tandem repeat analysis (MLVA) has been used successfully to genetically characterize *M. hyopneumoniae* isolates (Charlebois et al., 2014; Dos Santos et al., 2015; Nathues et al., 2011; Vranckx et al., 2011; Vranckx et al., 2012). This technique has a high discriminatory power, and can be applied directly to clinical samples without the necessity to grow the bacterium, which is very fastidious in the case of *M. hyopneumoniae* (Vranckx et al., 2011).

Previous studies have shown that there is a high diversity of *M. hyopneumoniae* field isolates, especially between strains from different herds (Charlebois et al., 2014). Other studies including a limited number of herds not practising vaccination against *M. hyopneumoniae*, showed that in some herds, only one strain was detected, whereas different strains were found in other herds, even in the same pig (Vranckx et al., 2011; Vranckx et al., 2012). The importance of genetic diversity of *M. hyopneumoniae* strains however is not fully known. A possible link between the presence of multiple simultaneous or subsequent infections with different *M. hyopneumoniae* strains and the presence and severity of lung lesions has been suggested (Charlebois et al., 2014; Villarreal et al., 2009; Vranckx et al., 2011), but no systematic study has been conducted to answer this question. If the presence of different *M. hyopneumoniae* strains is associated with more clinical disease and/or lung lesions, then measures decreasing the diversity of strains may be helpful to control respiratory problems in pig herds.

The aim of this study was to investigate the presence of different *M. hyopneumoniae* strains in consecutive batches of slaughter pigs from different herds, to type the strains using MLVA and to investigate associations between the occurrence of multiple strains of *M. hyopneumoniae* and the prevalence and severity of lung lesions.

## Materials and methods

### Study population

A list of herds (n=56) complying with following criteria: closed herd or closed production system, herd with at least 100 breeding sows and vaccination of piglets against *M. hyopneumoniae* was provided by one of the largest slaughter houses in Belgium (Covalis). The list of these farms was randomised (Excel 2010, Microsoft Corp., Redmond, WA) and the farmers were contacted in order of appearance on the randomized list until ten herds willing to participate to the study were obtained. Descriptive data of the ten study herds are presented in table 1.

**Table 1: Description of the ten study herds (A-J) enrolled in the study**

| Herd | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| **Number of sows** | 170 | 200 | 250 | 200 | 150 | 250 | 200 | 250 | 150 | 125 |
| **Sows breed** | LW (50%) + ELR (25%) + FLR (25%) | Topigs | ELR (50%) + FLR (50%) | Danbred (70%) + Hypor | Topigs | Topigs | Danbred | Rattlerow - Seghers | Hypor (90%) + Danbred (10%) | Hypor |
| **Batch production system for sows** | 3-week | 3-week | 4-week | 3-week | Continous | 1-week | 4-week | 1-week | 4-week | 4-week |
| **Stocking density nursery ($m^2$/animal)** | >0.30 | <0.30 | <0.30 | >0.30 | <0.30 | >0.30 | <0.30 | >0.30 | >0.30 | <0.30 |
| **Stocking density fatteners ($m^2$/animal)** | >0.70 | >0.7 | 0.65-0.70 | 0.65-0.7 | 0.7 | <0.65 | 0.65-0.70 | 0.65-0.70 | 0.65-0.70 | 0.65 |
| **Purchase of gilts (occasions per year)** | no | yes (5) | no | yes (4) | yes (4) | no | no | yes (8) | Yes Every month (*) | yes (5) |
| **Duration of quarantine period for gilts** | n.a. | 9 week | n.a. | 8 week | 4 week | n.a. | n.a. | no | 4 week | 6 week |
| ***M. hyopneumoniae* vaccination gilts** | no | yes | no | yes | no | no | no | no | yes | no |
| ***Age (days) at vaccination of piglets against M. hyopneumoniae*** | 8 and 26 | 21 | 14 | 7 | 14 | 7 | 35 | 3-8 and 28 | 18 | 21 |
| **Other vaccinations in piglets** | no | PCV-2 (21) | no | PRRSv (18) | PCV-2 (14) | no | PCV-2 (35) | no | PCV-2 (18) | no |
| **Age at weaning** (days of age) | 26 | 26 | 21 | 23 | 25 | 24 | 19 | 28 | 20-21 | 21 |
| **Clinical signs of *M. hyopneumoniae*** | no | no | no | yes | yes | no | no | no | no | no |
| **Coughing score for fattening pigs provided by the farmer (0-10)** | 3 | 2 | 0 | 7 | 4 | 2 | 3 | 0 | 1 | 0 |

LW = large white

ELR = English land race

FLR = French land race

n.a. = not applicable

PCV-2= Porcine Circovirus type 2

PRRSv = Porcine reproductive and respiratory syndrome virus

(*) schedule of purchasing gilts has been accelerated with transition to Danbred

Different potential risk factors for respiratory disease were collected from these herds during a herd visit by the first author. During the visit, a questionnaire was completed, the stables were visited and the fattening pigs inspected. The potential risk factors in the questionnaire were based on previous studies (Villarreal, 2010) and pertained to biosecurity, management, housing and vaccination status (table 2).

**Table 2: Potential risk factors for respiratory disease that were collected from the ten herds**

| | |
|---|---|
| **Continuous variables** | |
| Times per year farmer is purchasing gilts | 'X' times per year that the farmer purchased gilts |
| Number of herds surrounding the herd in a perimeter of < 5 km | calculated with Lambert coordinates and the Pythagoras Theorem |
| Number of sows present on the herd | Measure for the size of the herd |
| Production system for the sows | 0: no week system, 1, 2, 3, 4 - week system |
| Coughing score given by the farmer | (0-10) fatteners |
| **Categorical variables** | |
| Purchase of gilts | 1 = yes, 0 = no |
| Purchase of gilts always from the same supplier | 1 = yes, 0= no |
| Quarantine period for gilts | 1 = yes, 0 = no |
| Herd located close to a highway (< 5 km) | 1 = yes, 0 = no |
| Herd located near a slaughter house (<5 km) | 1 = yes, 0 = no |
| Distance herd to the public road (< 100 or > 100 m) | 1 ( < 100 m), 2 ( > 100 m) |
| Sow breed | 0: Topigs, 1: LW+ELR+FLR, ELR+FLR, Danbred+hypor, Danbred, RA-SE, Hypor |
| Dynamic or stable groups for pregnant sows | Stable (0) or dynamic (1) group sows |
| AIAO farrowing unit | 1 = yes, 0 = no |
| AIAO nursery unit | 1 = yes, 0 = no |
| AIAO fattening unit | 1 = yes, 0 = no |
| Stocking density nursery | 1 < 0.30 $m^2$/pig; 2 > or = 0.30 $m^2$/pig |
| Cross fostering during first week of life piglets | 0 = no, 1 < 10%, 2 = > 10% |
| Cross fostering after first week of life piglets | 0 = no, 1<10%, 2=>10% |
| Stocking density fattening unit | 1 = >0.70 $m^2$/pig , 2 = 0.70- 0.65 $m^2$/pig, 3 < 0.65 $m^2$/pig |
| Cleaning and disinfection farrowing unit | 1 = yes, 0 = no |
| Cleaning and disinfection nursery | 1 = yes, 0 = no |
| Cleaning and disinfection fattening unit | 1 = yes, 0 = no, 2 = only cleaned not disinfected |
| Stand empty period farrowing unit | 1 = yes, 2 = not always 0= no |
| Stand empty period nursery unit | 1 = yes, 2 = not always, 0 = no |
| Stand empty period fattening unit | 1 = yes, 2 = not always, 0 = no |
| Gilts vaccinated against *M.hyopneumoniae* | 1 = yes, 0 = no |
| Clinical signs of *M. hyopneumoniae* in grower-finishers | 1 = yes, 0 = no |

LW+ELR+FLR: large white, English landrace, French landrace

ELR+FLR: English landrace, French landrace

RA-SE: Rattlerow-Seghers

## Sampling at the slaughterhouse and lung lesion scoring

Three different batches of fattening pigs per herd were evaluated at the slaughterhouse during a time span of one to three months. All visits were performed from November 2012 until April 2013. From each batch, 20 randomly selected blood samples were collected at exsanguination, and from 20 other randomly selected pigs, the lungs were collected. For practical reasons, only the left half of the lung was taken. The blood samples and lungs were transported to the laboratory of Bacteriology of the Faculty of Veterinary Medicine, Ghent University immediately after the slaughterhouse visit.

Additionally, as many lungs as possible of each batch (min. 50) were evaluated for lung lesions. The lungs that were sampled were not included in the lung lesion scoring. The lungs were scored for presence of pneumonia and severity of *Mycoplasma*-like lesions using the method described by Morrison et al. (1985). *Mycoplasma*-like lesions were defined as macroscopic greyish to purplish consolidated pneumonia areas, generally located on the cranio-ventral parts of the lung lobes. The lungs were also evaluated for the presence of fissures and pleurisy. Fissures were defined as areas of collapsed alveoli adjoining alveolar emphysema (recovery lesions) (Kobish et al., 1993), while pleurisy was defined as fibrotic adherences between the parietal and visceral membranes of the pleural cavity (Chapter 3.2). No approval of the ethical committee of Ghent University was necessary, as the pigs were destined for slaughter.

## Nested Polymerase Chain Reaction (nPCR)

Upon arrival in the laboratory, the lung halves were flushed with 20 ml phosphate buffered saline (PBS, 8 g/L NaCl, 0.34 g/L $KH_2PO4$, 1.21 g/L $K_2HPO_4$, pH 7.3). The recovered fluid was centrifuged at 2000 *g* during 30 minutes to obtain the remaining pellet by carefully removing the supernatant. The pellet was resuspended in 1 ml of PBS and 200 µl of the resuspension was used to perform the DNA extraction using the DNeasy blood and tissue kit (QIAGEN, Belgium) according to the instructions in the protocol manual. *Mycoplasma hyopneumoniae* - DNA was detected with a two steps nested polymerase chain reaction (nPCR) (Stärk et al., 1998). The nPCR products were analyzed by gel electrophoresis on a 1.5% agarose gel in Tris-Borate-EDTA (TBE)-buffer and stained with GelRed™ (Biotium. Inc., CA. USA) with visualization under UV illumination.

## Multiple Locus Variable Number Tandem Repeat Analysis (MLVA)

All nPCR positive samples were submitted to a multiplex PCR as previously described (Vranckx et al., 2011). Briefly, loci h1, h5 repeat 2, p97 repeat 1 and p146 repeat 3 were amplified in a multiplex reaction with a mastercycler epgradient S (Eppendorf, Hamburg, Germany) in a final volume of 20 µl containing 1X PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 3 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 0.75 U of Platinum® *Taq* DNA Polymerase (Invitrogen, Merelbeke, Belgium), 0.1 µM of each primer and finally 2 µl of template DNA. Ten cycles (30" 94°C; 30" 63°C; 1'15" 69°C) in which the annealing temperature was incrementally decreased with 1°C per cycle were performed. Next, forty cycles (30" 94°C; 30" 53°C; 1'15" 69°C) and a final extension step of five minutes at 69°C followed.

The PCR-products were diluted 1:10 with high performance liquid chromatography filtered water (HPLC-H2O). Amplicons were kept at 4 °C for a maximum of 48 hours. A volume of 165 µl Hi-Di formamide (one run, 16 samples) (Applied Biosystems, Halle, Belgium) or a multitude of 165 µl for multiple runs was pipetted in an 1.5 ml Eppendorf tube (Eppendorf Belgium N.V.- S.A, Rotselaar, Belgium) and 1.5 µl of 600 LIZ standard (Applied Biosystems, Halle, Belgium) was added. Ten µl of this mixture was added to one µl of sample (PCR-product). Samples were denatured at 95°C for 5 min, cooled on ice and electrophoresis was applied on the ABI 3130xl genetic analyzer (Applied Biosystems) for 16 samples at 15kV during 14000 seconds at 65°C or for more than 16 samples on the ABI 3730xl (Applied Biosystems) at 15kV during 14000 seconds at 70°C.

The resulting electropherogram files were imported into BioNumerics version 7.5 (Applied Maths, Sint-Martens-Latem, Belgium). After normalization, the VNTR numbers were calculated automatically from the detected peaks. A minimal spanning tree was constructed with the Prims' algorithm using the multistate categorical coefficient. Only samples for which all four loci were detected, were included in the tree. A weight factor was assigned to each locus according to its' allelic variation in the obtained dataset with the highest weight assigned to the locus with the lowest variation. Following weights were assigned to each locus: 2, 3, 3 and 6 to p146, h1, h5, and p97, respectively. A strain was defined as a unique MLVA-type, *e.g.* if the combination of repeat numbers was unique. Clonal complexes were defined when strains differed in no more than one locus, with the exception of the most stable locus p97. The Hunter-Gaston discriminatory index was calculated for the complete dataset, as well as for each herd (Hunter and Gaston, 1988).

## Serology

The sera of the blood samples (20 per batch) were tested for presence of antibodies against *M. hyopneumoniae* using a blocking ELISA (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit, Oxoid Limited, Hampshire, UK). Sera with optical density (OD) <50% of the average value of the OD-buffercontrol were considered to be positive (ELISA *M. hyopneumoniae* positive samples). All values above or equal to 65% of the average value of the OD-buffercontrol were classified as negative. All doubtful samples equal to 50% and less than 65% of the average value of the OD-buffercontrol were considered to be negative as well.

Eight of the 20 samples from each batch were also tested for presence of antibodies against porcine reproductive and respiratory syndrome virus (PRRSv) (HerdCheck PRRS X3, IDEXX, Liebefeld-Bern, Switzerland) and subtypes H1N1, H1N2 and H3N2 SIV (standard haemagglutination-inhibition test).

## Statistical analyses

Different statistical models were used to assess the associations between the number of strains on the one hand and the presence and severity of lung lesions on the other hand. The number of different strains found in each batch of pigs was categorized as follows: category 1 (CAT 1): one *M. hyopneumoniae* strain per batch, category 2 (CAT 2): two to six different strains per batch, and category 3 (CAT 3): ≥ seven different strains per batch. The category one strain per batch was used as reference; the classification in category 2 and 3 was made to obtain the same number of strains in these categories.

The number of strains per batch was considered as explanatory variable in the models. As lung lesions may not only be caused by infection with *M. hyopneumoniae* and/or determined by the number of strains, the effect of the different potential risk factors for respiratory disease (table 2) was also taken into account in the models. A forward selection procedure was used during the model building, and risk factors with a P-value >0.15 were removed. Remaining risk factors (with P-value <0.15) were tested for collinearity. Correlations were assessed using Pearson's (continuous variables) or Spearman rank (categorical variables) correlation, and in case two variables were highly correlated ($|r| > 0.6$), the most significant factor was retained. In the final model, only risk factors with a P-value < 0.05 were retained. Confounding factors were identified when the regression coefficient ($\beta$) of another risk factor deviated more than 25% or 0.1 when $\beta < 0.4$ when removing the factor from the model. Such factors were excluded, but mentioned below each model. In total, four separate multivariable models were tested. The outcome variables for the different models were: severity of *Mycoplasma*-like lesions, likelihood of pneumonia lesions, fissures and pleurisy. Ln-transformation of the severity of the *Mycoplasma*-like lesions was performed to normalize the data. In all models, herd and lung were included as a random effect and batch was included as fixed effect.

A linear mixed regression model (MLwiN 2.26 (Rasbash et al., 2012)) was used to assess the influence of category of number of strains on the severity of the *Mycoplasma*-like lesions in each batch. The assumptions of normality and homogeneity of variance of the final model were tested by examining normal probability plots of residuals and plots of residuals versus predicted values. No patterns indicating heteroscedasticity were present. The multilevel linear regression model may be represented mathematically as: $Y_{ij} = \beta_0 + \beta_1 \text{category } 2_{ij} + \beta_2 \text{category } 3_{ij} + \text{batch}2_{ij} + \text{batch}3_{ij} + \varepsilon_{ij}$, where $Y_{ij}$ is the continuous outcome variable (severity of *Mycoplasma*-like lesions), $\beta$s are the model coefficients, category is the fixed effect of the category of different number of strains, batch is the fixed effect of batch 1 to 3, herd is the random effect of herd *i* (*i* = 1 to 10), j refers to the jth lung in the ith herd and $\varepsilon_{ij}$ is the random error term, assumed to be normally distributed with mean 0 and variance $\sigma^2$.

Logistic mixed regression models using 1st order marginal quasi-likelihood algorithms were used to assess the influence of strain category on the likelihood of pneumonia, fissures and pleurisy (MLwiN 2.26 - Centre for Multilevel Modeling, Bristol, UK (Rasbash et al., 2012)). The fit of the models was evaluated by inspection of the lung standardized residuals plotted against the normal scores and the lung level predicted values. The Hosmer Lemeshow goodness-of-fit measure was calculated for the explanatory variable models using SAS 9.3 (PROC LOGISTIC, SAS Institute Inc., NC, USA). The results were represented as odds ratio (OR) with the 95% confidence interval calculated around these odds ratios. The multilevel logistic regression model may be represented mathematically as: $g(Y_{ij}) = \beta_0 + \beta_1 \text{category } 2_{ij} + \beta_2 \text{category } 3_{ij} + \text{batch}2_{ij} + \text{batch}3_{ij} + \varepsilon_{ij}$, where (g) refers to the logit link function, $Y_{ij}$ is the probability of the outcome variable on the logit scale (likelihood of pneumonia, fissures and pleurisy), $\beta$s are the model coefficients, category is the fixed effect of category of number of strains, batch is the fixed effect of batch 1 to 3, herd is the random effect of herd *i* (*i* = 1 to 10), j refers to the jth lung in the ith herd and $\varepsilon_{ij}$ is the random error term, assumed to be normally distributed with mean 0 and variance $\sigma^2$.

# Results

## Descriptive results of the nPCR, MLVA, lung lesions and serology

*Nested PCR*

From the 600 bronchoalveolar fluid samples, 495 (82.5%) tested positive using nPCR for *M. hyopneumoniae*. The average percentage of positive samples in each category (table 3) per batch were: CAT 1: 42.5%, CAT 2: 79.6% and CAT 3: 91.1%. In all batches of each herd, nPCR positive samples were detected. The descriptive nPCR results for each herd and for each batch per herd separately are shown in table 4.

**Table 3: Descriptive results in the three category groups: prevalence of nPCR positive results, average number of different strains, severity of *Mycoplasma*-like lesions±SD, prevalence pneumonia, fissures and pleurisy expressed in percentages**

| | Category | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | Overall |
| **nPCR results** | 42.5 | 79.6 | 91.1 | 82.5 |
| **Average number of different strains** | 1 | 4 | 9 | 7 |
| **Severity of *Mycoplasma*-like lesions±SD** | 0.78±2.4 | 3.97±10.7 | 5.54±12.7 | 4.59±11.7 |
| **Prevalence of pneumonia** | 11.8 | 23.2 | 29.7 | 25.9 |
| **Prevalence of fissures** | 29.2 | 41.4 | 42.3 | 41.3 |
| **Prevalence of pleurisy** | 10.2 | 21.2 | 29.1 | 24.6 |

Category: 1=batches with only one strain detected, 2= batches with 2-6 different strain and 3= batches with ≥ 7 strains detected

SD: standard deviation, n= number

nPCR results: nested polymerase chain reaction: percentage of positive animals for *M. hyopneumoniae*-DNA detected in the bronchoalveolar lavage fluid

Severity of *Mycoplasma*-like lesions: minimum 0% and maximum 100% of the lung surface affected with pneumonia

*Multiple locus variable number tandem repeat analysis (MLVA)*

Samples that were positive using nPCR were submitted to MLVA. In the entire dataset, 135 different *M. hyopneumoniae* strains were found (Figure 1). The Hunter-Gaston discriminatory index for the complete dataset and for each herd separately is presented in table 5. The average number of different strains per batch was 7 (min 1; max 13). The total number of strains and the number of different strains per batch are presented in table 4. The average number of different strains per batch in CAT 1, 2 and 3 were 1, 4 and 9, respectively (table 3).

**Table 4: Descriptive results of the strain data of ten herds and the three batches (1-3) within each herd:** prevalence of nPCR (n=600) positive results, number of different *M. hyopneumoniae* strains, total number of *M. hyopneumoniae* strains and number of bronchoalveolar lavage fluid samples obtained with detection of double or triple different strains.

| Herd | | A | B | C | D | E | F | G | H | I | J | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **nPCR** | | **88 (53/60)** | **63 (38/60)** | **87 (52/60)** | **80 (48/60)** | **95 (57/60)** | **83 (50/60)** | **98 (59/60)** | **100 (60/60)** | **63 (38/60)** | **66 (40/60)** | **83 (495/600)** |
| | **1** | 100 (20/20) | 55 (11/20) | 85 (17/20) | 95 (19/20) | 100 (20/20) | 95 (19/20) | 95 (19/20) | 100 (20/20) | 15 (3/20) | 5 (1/20) | 75 (149/200) |
| | **2** | 65 (13/20) | 55 (11/20) | 90 (18/20) | 90 (18/20) | 85 (17/20) | 90 (18/20) | 100 (20/20) | 100 (20/20) | 90 (18/20) | 95 (19/20) | 86 (172/200) |
| | **3** | 100 (20/20) | 80 (16/20) | 85 (17/20) | 55 (11/20) | 100 (20/20) | 65 (13/20) | 100 (20/20) | 100 (20/20) | 85 (17/20) | 100 (19/20) | 87 (174/200) |
| **Number of different strains** | | **16** | **6** | **10** | **19** | **18** | **23** | **14** | **15** | **12** | **7** | **135** |
| | **1** | 9 | 2 | 5 | 8 | 11 | 13 | 6 | 10 | 2 | 1 | 65 |
| | **2** | 9 | 3 | 6 | 9 | 8 | 11 | 9 | 6 | 8 | 4 | 71 |
| | **3** | 7 | 1 | 3 | 6 | 10 | 7 | 4 | 5 | 3 | 3 | 49 |
| **Number of strains** | | **67** | **11** | **66** | **49** | **94** | **46** | **69** | **53** | **23** | **18** | **496** |
| | **1** | 25 | 5 | 15 | 18 | 32 | 17 | 21 | 20 | 2 | 1 | 156 |
| | **2** | 19 | 4 | 30 | 21 | 29 | 20 | 26 | 19 | 15 | 8 | 191 |
| | **3** | 23 | 2 | 21 | 10 | 33 | 9 | 22 | 14 | 6 | 9 | 149 |
| **BALF double strains** | | **16** | **0** | **15** | **4** | **36** | **11** | **13** | **6** | **1** | **0** | **102** |
| | **1** | 6 | 0 | 1 | 0 | 12 | 5 | 4 | 3 | 0 | 0 | 31 |
| | **2** | 5 | 0 | 10 | 3 | 10 | 4 | 5 | 2 | 1 | 0 | 40 |
| | **3** | 5 | 0 | 4 | 1 | 14 | 2 | 4 | 1 | 0 | 0 | 31 |
| **BALF triple strains** | | **1** | **0** | **1** | **0** | **1** | **2** | **1** | **0** | **0** | **0** | **6** |
| | **1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **2** | 1 | 0 | 1 | 0 | 1 | 2 | 1 | 0 | 0 | 0 | 6 |
| | **3** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Where applicable the prevalence data are followed by the number of positive samples (nPCR) / total number of samples (nPCR) between brackets

nPCR: nested polymerase chain reaction

BALF: Bronchoalveolar lavage fluid

1,2,3: respectively 1st, 2nd and 3rd batch of each herd

**Figure 1. Minimal spanning tree of all samples with a full MLVA profile in this study.** Samples in one colour belong to the same herd (A –J) and samples in a different shade of colour (1-3) belong to one of the three batches of each herd. Each circle represents a strain, the size of the circle is proportional with the number of samples belonging to a certain strain. Samples belonging to the same clonal complex, are marked with a grey background.

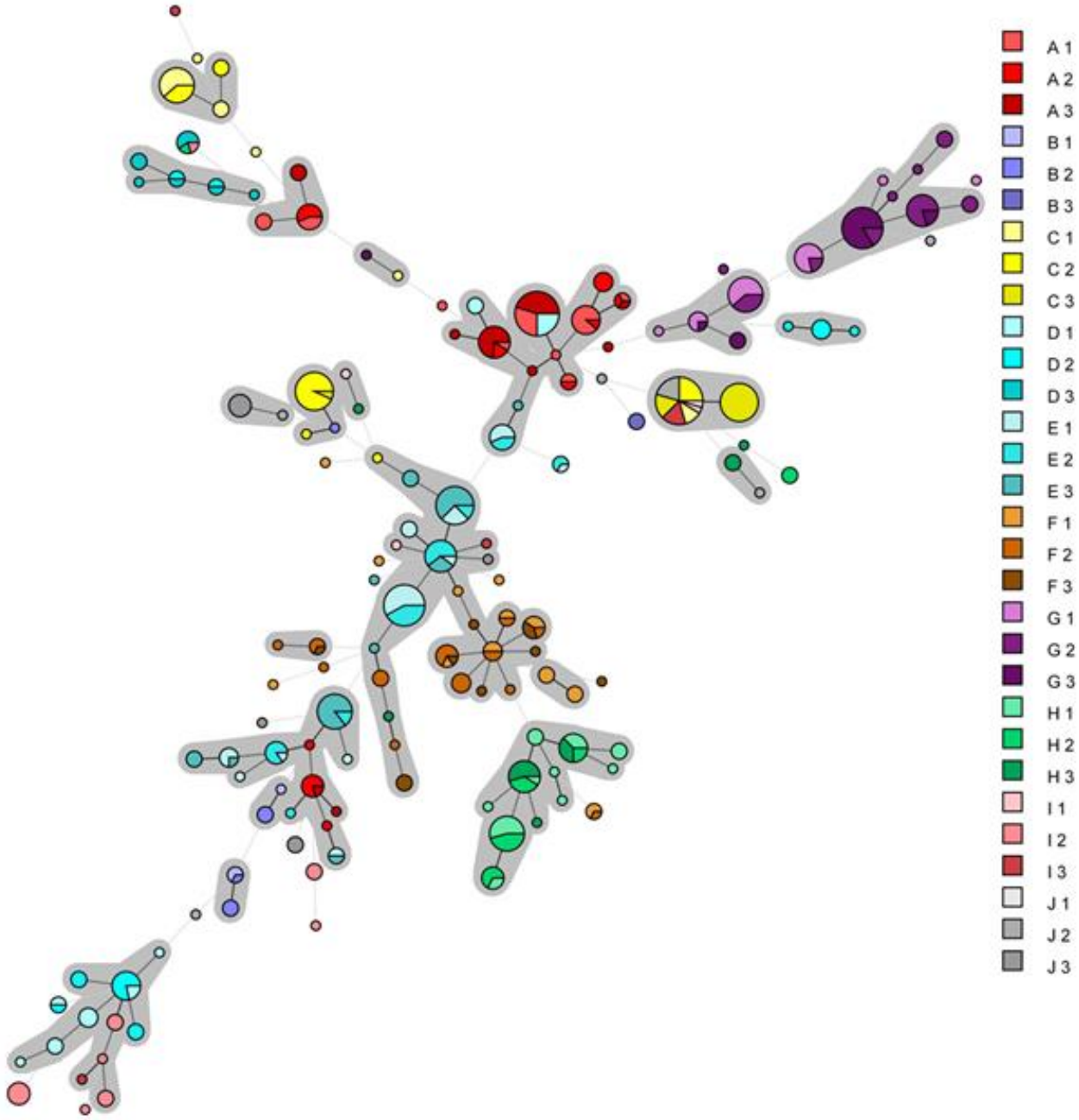

**Table 5: The Hunter-Gaston discriminatory index was calculated for the complete dataset, for each VNTR, as well as for each of the 10 herds** (A, B, C, D, E, F, G, H, I and J).

| | Hunter-Gaston Discriminatory index | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Herd** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** |
| | 89.7 | 89.1 | 82.0 | 93.8 | 89.1 | 95.7 | 85.8 | 88.2 | 92.3 | 85.2 |
| **VNTR** | **h1** | **h2** | **p146** | **p97** | **Total** | | | | | |
| | 88.0 | 88.5 | 90.8 | 77.5 | 98.4 | | | | | |

h1, h2, p146, p97: four VNTRs in the genome of *M. hyopneumoniae* of which the length of the amplified fragments were measured
Total: the Hunter-Gaston DI calculated for the entire dataset

The most prominent strain was strain 2, with 24 detections in the whole data set of all herds. This strain was detected in herds C, I and J. Strain 113 was the second most prominent strain and was found in herd A and D for a total of 20 times. Strain 135 was only detected 5 times in the dataset, however in herd D, H and I. Hundred and ten strains out of 135 were only detected 5 times or less and 60 out of 135 strains were only detected once. Strain 2, 42, 45, 59, 61, 77, 78, 109 and 117 were found in each of the three sampling periods in herds C, F, F, E, E, H, H and A respectively. In herds B, D, G, I, J no strains were found circulating throughout all three sampling periods. In all herds, strains were identified that were detected in at least two out of three sampling periods, except for herd B. Most returning strains per batch were found in two consecutive sampling points. Five strains were found in the first and the third sampling point only: strain 2, 19, 21, 99 and 113 in respectively herd I, E, E, G and A. In a lot of lungs (102), two and a few lungs (6) three different strains were detected (table 4). In herds B and J, no samples with two different strains were obtained. In two lungs of herd F, three different strains were found. In herds A, C, E, and G, always in the second batch, one sample with detection of three strains was found. In herds B, D, H, I and J no samples with three strains were detected.

*Lung lesions*

In total, 3820 lungs were evaluated at the slaughter line. The average (min.-max.) number of lungs scored per herd and per batch were 382 (200-494) and 127 (54-186), respectively. The average severity of *Mycoplasma*-like lesions in CAT 1, 2 and 3 were 0.78±2.4%, 3.97±10.7 and 5.54±12.7. The average prevalence of pneumonia was 11.8, 23.2 and 29.7% in CAT 1, CAT 2 and CAT 3, respectively. The average prevalence of fissures was 29.2, 41.4 and 42.3% in CAT 1, CAT 2 and CAT 3, respectively. The average prevalence of pleurisy in CAT 1, CAT 2 and CAT 3 was 10.2, 21.2 and 29.1%, respectively (table 3). The severity scores of *Mycoplasma*-like lesions, and the prevalence of pneumonia, fissures and pleurisy of each herd and each batch are shown in table 6.

**Table 6: Descriptive results of lung lesions in the ten herds (A-J) and the three batches (1, 2, 3) within each herd:** severity of *Mycoplasma*-like lesions (n=3605), prevalence of pneumoniae (n=3605), fissures (n=3605) and pleurisy (n=3820).

| Herd | | A | B | C | D | E | F | G | H | I | J | Overall |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Mycoplasma*-like lesions** | | **7.2 ± 12.6** | **0.6 ± 1.9** | **4.8 ± 11.5** | **1.8 ± 5.1** | **10.2 ± 17.3** | **2.7 ± 9.0** | **7.8 ± 14.4** | **3.9 ± 10.8** | **4.0 ± 11.6** | **2.3 ± 7.7** | **4.6 ± 11.6** |
| | **1** | 7.3 ± 12.2 | 0.6 ± 1.9 | 1.7 ± 3.4 | 2.1 ± 4.2 | 5.9 ± 10.1 | 0.4 ± 1.4 | 6.4 ± 12.8 | 6.7 ± 13.8 | 2.1 ± 10.3 | 1.2 ± 2.9 | 3.6 ± 9.3 |
| | **2** | 8.3 ± 12.7 | 0.6 ± 1.9 | 4.1 ± 9.2 | 1.3 ± 4.7 | 10.9 ± 17.9 | 0.9 ± 5.7 | 6.7 ± 14.0 | 2.1 ± 7.6 | 1.4 ± 6.0 | 0.1 ± 0.9 | 3.6 ± 10.5 |
| | **3** | 6.3 ± 13.0 | 0.5 ± 1.9 | 8.6 ± 16.7 | 1.8 ± 5.9 | 13.2 ± 20.5 | 6.6 ± 13.3 | 10.4 ± 15.9 | 2.5 ± 9.1 | 7.9 ± 15.2 | 4.9 ± 11.5 | 6.4 ± 14.0 |
| **Pneumonia %** | | **42 (83/197)** | **9 (29/310)** | **30 (69/229)** | **19 (81/432)** | **45 (205/455)** | **17 (70/422)** | **41 (179/433)** | **22 (91/407)** | **19 (71/382)** | **16 (54/338)** | **26 (932/3605)** |
| | **1** | 41 | 10 | 22 | 26 | 42 | 7 | 38 | 37 | 11 | 17 | 26 |
| | **2** | 48 | 10 | 30 | 13 | 45 | 4 | 35 | 16 | 7 | 2 | 20 |
| | **3** | 38 | 8 | 38 | 17 | 48 | 37 | 51 | 12 | 35 | 28 | 32 |
| **Fissures %** | | **46 (90/197)** | **32 (99/310)** | **34 (78/229)** | **38 (164/432)** | **48 (217/455)** | **37 (155/422)** | **39 (170/433)** | **52 (213/407)** | **51 (194/382)** | **32 (107/338)** | **41 (1487/3605)** |
| | **1** | 29 | 31 | 50 | 48 | 28 | 22 | 31 | 34 | 38 | 25 | 33 |
| | **2** | 33 | 33 | 17 | 32 | 54 | 30 | 44 | 66 | 46 | 33 | 41 |
| | **3** | 71 | 32 | 42 | 34 | 59 | 57 | 42 | 57 | 63 | 34 | 49 |
| **Pleurisy %** | | **15 (30/200)** | **8 (25/319)** | **29 (73/250)** | **35 (162/461)** | **37 (185/494)** | **27 (119/445)** | **16 (71/454)** | **33 (149/445)** | **29 (120/411)** | **2 (6/341)** | **25 (940/3820)** |
| | **1** | 1 | 2 | 7 | 39 | 38 | 21 | 16 | 25 | 32 | 3 | 21 |
| | **2** | 26 | 7 | 45 | 35 | 37 | 28 | 20 | 34 | 27 | 2 | 27 |
| | **3** | 20 | 15 | 26 | 32 | 37 | 32 | 11 | 43 | 30 | 1 | 26 |

Where applicable the prevalence data are followed by the number of positive results (prevalence lung lesions) / total number of lungs scored (lung lesions) between brackets

SD: standard deviation, n= number

*Mycoplasma*-like lesions: minimum 0% and maximum 100% of the lung surface affected with pneumonia

1,2,3: respectively 1st, 2nd and 3rd batch of each herd

Number of lungs scored for severity of *Mycoplasma*-like lesions, prevalence of pneumonia and fissures: 3605 (because of severe pleurisy in some lungs it was not possible to evaluate all lungs entirely)

Number of lungs scored for prevalence of pleurisy: 3820

**Table 7: Seroprevalence of *M. hyopneumoniae*, PRRSV, swine influenza subtypes H1N1, H1N2 and H3N2 in the ten herds (A-J)**

| Herd | A | B | C | D | E | F | G | H | I | J | Overall |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **ELISA *M. hyopneumoniae* n=600** | 85 (51/60) | 33 (20/60) | 90 (54/60) | 77 (46/60) | 100 (60/60) | 98 (59/60) | 97 (58/60) | 87 (52/60) | 20 (12/60) | 48 (29/60) | 74 (441/600) |
| **ELISA PRRSv n=240** | 100 (24/24) | 96 (23/24) | 96 (23/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 99 (238/240) |
| **HI influenza H1N1 n=240** | 88 (21/54) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 79 (19/24) | 92 (22/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 96 (230/240) |
| **HI influenza H1N2 n=240** | 96 (23/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (24/24) | 100 (239/240) |
| **HI influenza H3N2 n=240** | 54 (13/24) | 71 (17/24) | 67 (16/24) | 42 (10/24) | 75 (18/24) | 71 (17/24) | 54 (13/24) | 21 (5/24) | 54 (13/24) | 79 (19/24) | 59 (141/240) |

Seroprevalence data are followed with number of positive samples / total number of samples between brackets

n= number

PRRSv: porcine reproductive

HI: hemagglutination inhibition titers

SIV: swine influenza virus

*Serology*

The serological results for *M. hyopneumoniae*, PRRSv and H1N1, H1N2, H3N2 SIV of each herd and each batch per herd are shown in table 7.

*Association between diversity of M. hyopneumoniae strains and lung lesions*

The results of the final multivariable models are shown in table 8. The severity of *Mycoplasma*-like lesions and the prevalence of pneumonia were higher in batches of CAT 2 than in batches of CAT 1 and significantly higher in batches of CAT 3 than in batches of CAT 1 (P= 0.064 for CAT 2 to CAT  and P= 0.012 for CAT 3 to CAT 1 and OR: 1.30; P=0.33 for CAT 2 to CAT 1 and OR: 2.08; P=0.012 for CAT 3 to CAT 1, respectively for the severity of *Mycoplasma*-like lesions and the prevalence of pneumonia).
In batches of CAT 2 and 3, there was a higher prevalence of fissures than in batches of CAT 1: CAT 2 to CAT 1: OR= 1,35; P=0.094 and CAT 3 to CAT 1: OR= 1.70; P=0.007).
Batches belonging to CAT 2 and 3 showed a lower prevalence of pleurisy (overall P<0.001, CAT 2- CAT 1: OR= 0.35; P<0.001 and CAT 3 to CAT 1: OR= 0.34; P<0.001).

**Table 8a: Results of the four final multivariable models, with** severity of *Mycoplasma*-like lesions, prevalence of pneumonia, fissures and pleurisy as outcome variables. For severity of *Mycoplasma*-like lesions, a linear model was used. For the other outcome variables, a logistic model was used. For category (CAT), CAT 1 was the reference, for Batch, Batch 1 was the reference.

| **Severity of *Mycoplasma*-like lesions** | **β** | **SE** | **OR** | **$CI_{min}$** | **$CI_{max}$** | *P* |
|---|---|---|---|---|---|---|
| **Intercept** | -2.85 | 0.18 | - | - | - | |
| **CAT** | | | | | | 0.027 |
| CAT 2 | 0.35 | 0.19 | - | - | - | 0.064 |
| CAT 3 | 0.51 | 0.20 | - | - | - | 0.012 |
| **Batch** | | | | | | < 0.001 |
| Batch 2 | 0.16 | 0.07 | - | - | - | 0.027 |
| Batch 3 | 0.38 | 0.07 | - | - | - | 0.0021 |
| **Distance to public road[A]** | | | | | | 0.0015 |
| <100 m | | | | | | |
| >100 m | 0.24 | 0.08 | - | - | - | 0.0015 |
| **Stand empty farrowing unit[B]** | | | | | | < 0.001 |
| no | | | | | | |
| yes | -0.31 | 0.08 | - | - | - | < 0.001 |
| not always | 0.03 | 0.09 | - | - | - | 0.70 |

| **Likelihood of pneumonia** | **β** | **SE** | **OR** | **$CI_{min}$** | **$CI_{max}$** | *P* |
|---|---|---|---|---|---|---|
| **Intercept** | -2.24 | 0.34 | 0.11 | 0.05 | 0.21 | |
| **CAT** | | | | | | < 0.001 |
| CAT 2 | 0.26 | 0.27 | 1.30 | 0.77 | 2.19 | 0.33 |
| CAT 3 | 0.73 | 0.29 | 2.08 | 1.18 | 3.68 | 0.012 |
| **Batch** | | | | | | < 0.001 |
| Batch 2 | -0.45 | 0.11 | 0.64 | 0.52 | 0.78 | < 0.001 |
| Batch 3 | 0.42 | 0.10 | 1.53 | 1.25 | 1.86 | < 0.001 |
| **Number of herds surrounding the trial herd in a perimeter < 5 km** | 0.01 | 0.00 | 1.01 | 1.01 | 1.01 | < 0.001 |
| **Vaccination gilts *M. hyopneumoniae*[B]** | | | | | | < 0.001 |
| no | | | | | | |
| yes | -0.99 | 0.23 | 0.37 | 0.24 | 0.58 | < 0.001 |

| **Likelihood of fissures** | **β** | **SE** | **OR** | **$CI_{min}$** | **$CI_{max}$** | *P* |
|---|---|---|---|---|---|---|
| **Intercept** | -0.94 | 0.20 | 0.39 | 0.26 | 0.58 | |
| **CAT** | | | | | | 0.008 |
| CAT 2 | 0.30 | 0.18 | 1.35 | 0.95 | 1.93 | 0.094 |
| CAT 3 | 0.53 | 0.20 | 1.70 | 1.15 | 2.50 | 0.007 |
| **Batch** | | | | | | < 0.001 |
| Batch 2 | 0.29 | 0.09 | 1.34 | 1.12 | 1.59 | 0.001 |
| Batch 3 | 0.74 | 0.09 | 2.09 | 1.75 | 2.48 | < 0.001 |
| **Distance to public road[A]** | | | | | | 0.009 |
| <100 m | | | | | | |
| >100 m | 0.26 | 0.10 | 1.30 | 1.07 | 1.58 | 0.009 |
| **Stand empty farrowing unit[B]** | | | | | | < 0.001 |
| yes | -0.40 | 0.10 | 0.67 | 0.55 | 0.82 | < 0.001 |
| not always | -0.76 | 0.14 | 0.47 | 0.36 | 0.61 | < 0.001 |

**Table 8b: Results of the four final multivariable models, with** severity of *Mycoplasma*-like lesions, prevalence of pneumonia, fissures and pleurisy as outcome variables. For severity of *Mycoplasma*-like lesions, a linear model was used. For the other outcome variables, a logistic model was used. For category (CAT), CAT 1 was the reference, for Batch, Batch 1 was the reference.

| **Likelihood of pleurisy** | **β** | **SE** | **OR** | **$CI_{min}$** | **$CI_{max}$** | *P* |
|---|---|---|---|---|---|---|
| **Intercept** | 0.25 | 0.72 | 1.29 | 0.32 | 5.25 | |
| **CAT** | | | | | | < 0.001 |
| CAT 2 | -1.06 | 0.27 | 0.35 | 0.21 | 0.59 | < 0.001 |
| CAT 3 | -1.07 | 0.30 | 0.34 | 0.19 | 0.62 | < 0.001 |
| **Batch** | | | | | | 0.002 |
| Batch 2 | 0.35 | 0.10 | 1.41 | 1.16 | 1.72 | < 0.001 |
| Batch 3 | 0.25 | 0.11 | 1.29 | 1.04 | 1.59 | 0.021 |
| **Cross fostering piglets during first week of life[B]** | | | | | | 0.002 |
| no | | | | | | |
| <10% | -0.32 | 0.71 | 0.73 | 0.18 | 2.91 | 0.67 |
| >10% | -1.96 | 0.77 | 0.14 | 0.03 | 0.64 | 0.040 |

clinical signs *M. hyopneumoniae* with intensity mixing CF confounded

OR: odds ratio, CI: confidence interval, SE: standard error, P: p-value, Batch 1, 2, 3: referring to respectively the first, second and third sampling point in each herd, CAT (category) 1 (one *M. hyopneumoniae* strain per batch per herd), CAT 2: category 2 (two to six strains per batch per herd), CAT 3: category 3 (≥ seven strains per batch per herd), [A] < 100 km is reference category, [B] no is reference category

## Discussion

The present study revealed that, using MLVA, many different *M. hyopneumoniae* strains are present in slaughter pigs from different pig herds and batches within a herd. The results also showed that prevalence and severity of pneumonia lesions at slaughter were significantly higher in batches where more different *M. hyopneumoniae* strains were found.

The ten selected study herds can be considered as representative for other pig herds, as the housing, feeding and management practices are quite similar to most Belgian and West-European herds. Also the prevalence of lung lesions (pneumonia 26%, fissures 41%, and pleurisy 25%) was similar to the results of previous studies (Meyns et al., 2011). The fact that three different batches of pigs were investigated within a herd, allowed to account for possible variations over time within a herd.

The minimal spanning tree (MST) visualizes the phylogenetic relationship of the analysed strains. In comparison with previous work (Vranckx et al., 2012), the MST in the present study had a wide distribution, confirming the high diversity of the *M. hyopneumoniae* strains. A weighing factor was assigned to each locus according to its abundancy in the dataset. This allowed to take into account the importance of variation of less abundant loci. To the author's knowledge this is the first time this approach is used for analysing the diversity of an organism. The Hunter-Gaston discriminatory index (98.4 when all four VNTRs are combined), confirmed that MLVA is a suitable and discriminatory technique to investigate genetic differences in *M. hyopneumoniae* (Vranckx et al., 2011). The high variation in strains is also illustrated by the large number of different strains found at batch and even at animal level: in 102 pigs, two different strains were found, and in six pigs, three different strains were present. In theory more than three strains at animal level can be present and detected if multiple peaks in the electropherograms of each VNTR can be distinguished. In practice the MLVA-technique, has some limitations: the detection limit is 100 organisms/µl in bronchoalveolar lavage fluid and multiple strains can be detected if the differences in concentration are less than tenfold. Therefore, it cannot be excluded that only the dominant strains in the herd were detected (Vranckx et al., 2011). Although it is known from previous studies that pigs may be infected with more than one strain (Nathues et al., 2011; Vranckx et al., 2011; Vranckx et al., 2012), the results of the present study in vaccinated herds document a higher diversity of *M. hyopneumoniae* strains than shown by previous authors (Calus, 2010; Mayor et al., 2007; Nathues, 2011; Vranckx et al., 2012). The results also suggest that a high *M. hyopneumoniae* diversity might occur despite vaccination of piglets against *M. hyopneumoniae*. Some of the measures that might influence introduction of new strains in the farm might be purchasing and quarantine policy, swine density in the area, pig transport, all-in/all-out management and animal flow. It is not known whether contamination of the sampled pigs' lungs had occurred through the scalding water. Marois et al. (2008) showed that although *M. hyopneumoniae* was detected in the scalding water, the lungs of SPF pigs remained negative by nested PCR.

The prevalence and severity of pneumonia lesions at slaughter were significantly higher in batches where more different *M. hyopneumoniae* strains were found, illustrating for the first time the importance of strain diversity at batch level. The severity of *Mycoplasma*-like lesions, the prevalence of pneumonia and the prevalence of fissures was significantly higher in batches of CAT 3 compared to CAT 1, and numeric differences were obtained when

batches of CAT 2 were compared to CAT 1. The effect of batch was significant in all models, indicating that there is quite some variation between successive batches in a herd. It also indicates the importance of investigating more batches from each herd.

The exact explanation why more different *M. hyopneumoniae* strains at batch level may lead to more pneumonia lesions is not known. Some strains have been shown to be more virulent than others (Vicca et al., 2003), and infection with a low virulent strain did not protect against subsequent infection with a high virulent strain (Villarreal et al., 2009). On the contrary, clinical symptoms and lesions were more severe in case of dual infection. It is therefore possible that also at batch level, the presence of many different *M. hyopneumoniae* strains may lead to more (severe) pneumonia lesions. Further research to explain the mechanisms is necessary. Charlebois et al., (2014) did not find a significant association between the number of different *M. hyopneumoniae* strains and severity of lung lesions in slaughter pigs (Charlebois et al., 2014).

To account for infection pressure possibly influencing the lung lesion data, rather than the number of different strains, all models were run with nPCR results included in the model. Only in the pneumonia model, the factor nPCR needed to be retained, but the overall conclusions for each model, including the pneumonia model remained the same (data not shown). Apart from *M. hyopneumoniae*, also other respiratory pathogens may be involved in pneumonia lesions (Sibila et al., 2009). Almost all pigs tested for swine influenza and PRRS virus were positive, and therefore, it is unlikely that these pathogens have biased the results. As lung lesions are multifactorial, the effect of potential non-infectious risk factors was taken into account in the multivariable models (Del Pozo Sacristán, 2014; Stärk, 2000; Villarreal, 2010). This allowed to investigate the effect of strain diversity in batches, apart from the effect of these risk factors. As the aim of the study was mainly to assess the importance of strain diversity, the other significant risk factors in the final models will only be discussed briefly.

The severity of *Mycoplasma*-like lesions was higher in batches from herds located further away from a public road (more than 100 versus less than 100 meter), and when a stand-empty period in the farrowing unit was not practiced. The same two variables were also significant in the model for prevalence of fissures. One would expect that severity of lesions and prevalence of fissures to be higher in herds located closer to the public road, as this has been shown to be a risk factor for infection with *M. hyopneumoniae* (Stärk et al., 1992). One explanation could be that herds located further away from the public road are smaller herds with a lower biosecurity (Amass and Clark, 1999). Also, all herds were located quite close to a public road in the present study. Not practicing a stand-empty period can be considered as one aspect of poor hygiene and biosecurity, which has been shown as a risk factor for respiratory disease (Stärk, 2000).

The prevalence of pneumonia lesions was higher in case more other pig herds surrounded the herd, and when breeding gilts were not vaccinated against *M. hyopneumoniae*. Pig herd density in the region has been shown to be a risk factor for introduction of *M. hyopneumoniae* in the herd or for increased seroprevalence of *M. hyopneumoniae* (Maes et al., 2000; Villarreal, 2010). Purchasing gilts compared to no purchase was a risk factor for higher seroprevalence of *M. hyopneumoniae* in slaughter pigs (Maes et al., 2000). Younger sows are more likely to transmit the infection to their piglets (Fano et al., 2006) and vaccination of breeding sows may lead to a lower infection level in weaned pigs (Ruiz et al., 2003) and to a lower prevalence of pneumonia in slaughter pigs (Sibila et al., 2008).

Pleurisy was also measured in the study, as it is a common and economically important lesion. Experimental *M. hyopneumoniae* infection does however not lead to pleurisy lesions. Under field conditions, positive associations have been found between *M. hyopneumoniae* infection and pleurisy lesions (Meyns et al., 2011), although the results are not consistent (Fraile et al., 2010). In the present study, although the descriptive values showed a higher prevalence of pleurisy when comparing CAT 2 and CAT 3 with CAT 1, the final models resulted in a higher number of different *M. hyopneumoniae* strains being associated with a lower prevalence of pleurisy, though the effect was small. A high intensity of mixing and cross-fostering pigs (>10%) compared to no cross-fostering of piglets was associated with a lower prevalence of pleurisy. This might be explained by the fact that cross-fostering may lead to a better colostrum intake by the piglets, resulting in better performance and health during their lifetime (Declerck et al., 2016; Quesnel, 2011).

## Conclusions

MLVA testing on bronchoalveolar lavage fluid showed a high diversity of *M. hyopneumoniae* strains in slaughter pigs from herds vaccinated against *M. hyopneumoniae*. *Mycoplasma*-like lesions were more severe and the prevalence of pneumonia and fissures were higher when more different *M. hyopneumoniae* strains were present in a group of pigs. These results imply that inter- and intra-herd biosecurity measures decreasing the introduction of new *M. hyopneumoniae* strains, may lead to less (severe) pneumonia lesions in slaughter pigs.

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# *3.2. Impact of particulate matter and ammonia on average daily weight gain, mortality and lung lesions in pigs*

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## Abstract

The present study investigated the simultaneous influence of particulate matter ($PM_{10}$) and ammonia ($NH_3$) on performance, lung lesions and the presence of *Mycoplasma hyopneumoniae (M. hyopneumoniae)* in finishing pigs. A pig herd experiencing clinical problems of *M. hyopneumoniae* infections was selected. In total, 1095 finishing pigs of two replicates in eight compartments each were investigated during the entire finishing period (FP). Indoor $PM_{10}$ and $NH_3$ were measured at regular intervals during the FP with two Grimm spectrometers and two Graywolf Particle Counters ($PM_{10}$) and an Innova photoacoustic gas monitor ($NH_3$). Average daily weight gain (ADG) and mortality were calculated and associated with $PM_{10}$ and $NH_3$ during the FP. Nasal swabs (10 pigs/compartment) were collected one week prior to slaughter to detect DNA of *M. hyopneumoniae* with nested PCR (nPCR). The prevalence and extent of pneumonia lesions, and prevalence of fissures and pleurisy were examined at slaughter (29 wks). The results from the nasal swabs and lung lesions were associated with $PM_{10}$ and $NH_3$ during the FP and the second half of the FP.

In the univariable model, increasing $PM_{10}$ concentrations resulted in a higher odds of pneumonia lesions (second half of the FP: OR=8.72; P=0.015), more severe pneumonia lesions (FP: P=0.04, second half of the FP: P=0.009), a higher odds of pleurisy lesions (FP: OR=20.91; P<0.001 and second half of the FP: OR=40.85; P<0.001) and a higher number of nPCR positive nasal samples (FP: OR=328.00; P=0.01 and second half of the FP: OR=185.49; P=0.02). Increasing $NH_3$ concentrations in the univariable model resulted in a higher odds of pleurisy lesions (FP: OR=21.54; P=0.003) and a higher number of nPCR positive nasal samples (FP: OR=70.39; P=0.049; second half of the FP: OR=8275.05; P=0.01). In the multivariable model an increasing $PM_{10}$ concentration resulted in a higher odds of pleurisy lesions (FP: OR=8.85; P=0.049). These findings indicate that the respiratory health of finishing pigs was significantly affected by $PM_{10}$.

## Introduction

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the causative agent of enzootic pneumonia (EP) which is a chronic respiratory disease in swine (Maes et al., 2008). Lung lesions in grower-finishers caused by EP are still held responsible for tremendous losses to the pig sector (Maes et al., 2008; Meyns et al., 2011). Mycoplasmal pneumonia and pleurisy lesions have a negative impact on growth and feed conversion (Meyns et al., 2011). On average, 17% and 34% reduction in live weight gain and 14% and 26% increase in feed conversion can be attributed to mycoplasmal pneumonia and pleuropneumonia, respectively (Meyns et al., 2011; Straw et al., 1989).

Several management and environmental factors have been shown to contribute to enzootic pneumonia, such as low general herd health, poor disease prevention, high pen stocking density, and mixing pigs of different origin (Sibila et al., 2004; Stärk, 2000). Pigs are exposed to aerial pollutants, such as particulate matter (PM) and ammonia ($NH_3$) (Pedersen et al., 2000). Particulate matter is a mixture of suspended materials with particle-like properties, with different physical, chemical and biological characteristics, which determine its behavior and the associated environmental and health effects.

Similar to *M. hyopneumoniae*, which affects the mucosal clearance system by attaching to and disrupting the cilia on the epithelial surface of the respiratory tract (Thacker, 2006), PM and $NH_3$ have an adverse effect on the mucosal clearance system. Particulates cause inflammation or irritation of the respiratory epithelia (Pearson and Sharples, 1995). Ammonia depresses ciliary activity and mucus flow (Stombaugh et al., 1969). In this way, the function of the mucosal clearance system is impaired, thereby predisposing the respiratory tract to respiratory infections (Maes et al., 2008; Wilton et al., 1998). Several particle size ranges are often distinguished: total or inhalable particles, thoracal or fine particles and respirable particles. Total particles include all size particles (particles smaller than 50-100 μm). Thoracal particles are particulates ≤ 10 μm and can be inhaled into the respiratory tract of the pig (Aarnink and Ellen, 2007; Hinz, 2002; Jacobson et al., 2003; Takai et al., 1998). The majority of micro-organisms are present on particles sized 5-20 μm and mainly on particles of size 10 μm (Harry, 1978). Larger particles of approximately 5 μm or more will affect the mucociliary system (Collins and Algers, 1986). Respirable particles are smaller than 4 μm and will reach the alveoli (Collins and Algers, 1986; Hinz, 2002). Particulate matter ($PM_{10}$) can be defined as particulate matter which passes through a size-selective inlet with a 50% efficiency cut-off at 10 μm aerodynamic diameter (European Council, 1999). The abbreviation PM is mostly accompanied by an index number. This number refers to the size of the cut-off diameters of particle separators which separate particles from gaseous substances (Hinz, 2002).

Ammonia is the most important health threatening gas in animal houses (Cargill et al., 2002). Carbon dioxide ($CO_2$) and hydrogen sulfide ($H_2S$) are other gasses present in swine facilities. Carbon dioxide is less harmful to animal health (Cargill et al., 2002; Ni et al., 2008) whereas hydrogen sulfide can cause death in swine when manure pits are agitated (Stombaugh et al., 1969), but usually the concentrations in the facility are low (Jacobson et al., 2003). Other gasses detected in swine facilities are methane ($CH_4$) and nitrous oxide ($N_20$), however they are related to global climate changes (Jacobson et al., 2003; Ni et al., 2008). Total PM in various sections of the facilities throughout the year stay between 2000 and 3000 $\mu g/m^3$ (Lemay et al., 2002). The $PM_{10}$ concentration measured by Van Ransbeeck et al. (2013b) in a Flemish pig finishing stable during different seasons in different

ages of the pigs between 2009-2010 ranged from 22 to 2288 μg/m³ (Van Ransbeeck et al., 2013b). Ammonia levels range from five to ten ppm in well ventilated pig buildings and tend to be higher in stables with solid floors compared to those with slatted floors (Jacobson et al., 2003). Indoor $NH_3$ concentrations in finishing pig stables were 18.7 and 16.3 ppm on average for conventional and low emission stables respectively (Van Ransbeeck et al., 2013a).

To date, research focusing on the simultaneous impact of PM and $NH_3$ on specific respiratory diseases such as EP are scarce. Many studies focus on the impact of $NH_3$ or PM on atrophic rhinitis (AR), are dated and were performed in confined research facilities (Curtis et al., 1975; Done et al., 2005; Drummond et al., 1980; Hamilton et al., 1999; Hamilton et al., 1998; Stombaugh et al., 1969; Underdahl et al., 1982; Urbain et al., 1996; Wathes et al., 2004). No consensus is obtained on whether (Chiba et al., 1985; Curtis et al., 1975; Donham, 1991; Jolie et al., 1999b; Stombaugh et al., 1969) or not (Carpenter and Moulsley, 1986; Done et al., 2005; Drummond et al., 1980; Hamilton et al., 1999; Jansen and Feddes, 1995; Takai et al., 1995; Urbain et al., 1996; Van 't Klooster et al., 1993) PM or $NH_3$ affect health or production under current field conditions. Further, many studies consider only the impact of PM (Chiba et al., 1985; Jolie et al., 1999a; Van 't Klooster et al., 1993) or $NH_3$ (Hamilton et al., 1998; Stombaugh et al., 1969; Underdahl et al., 1982; Urbain et al., 1996) on respiratory parameters (Done et al., 2005; Hamilton et al., 1999; Hamilton et al., 1998; Urbain et al., 1996) or production data (Bate et al., 1987; Wathes et al., 2004), but not the associations and long term environmental exposures in relation with health and production. Only one study exposed pigs to aerial PM (40.6mg/m³) in combination with lipopolysaccharide for 15 weeks (5 days per week, 8 hours per day) but no influence on macroscopic lung lesions was noticed (Jolie et al., 1999a).

The present study investigated the simultaneous and long term influence of $PM_{10}$ and $NH_3$ on pig performance, lung lesions and the presence of *M. hyopneumoniae* under field conditions in a pig herd with clinical problems of enzootic pneumonia in grower-finishing pigs.

## Material and Methods

### Herd and animals

A commercial farrow-to-finish pig herd comprising 210 JSR (John Sykes Rymer Genetics Limited) hybrid sows with clinical respiratory problems associated with *M. hyopneumoniae* (*i.e.* dry coughing) in grower-finishing pigs was selected. Artificial insemination was performed with semen of Piétrain boars and the farm operated a four week batch production system. The piglets were vaccinated against *M. hyopneumoniae* at day three and day 21 with Suvaxyn Mhyo® (Zoetis). At weaning age (21 days), the pigs were transferred to the nursery unit (three pens, one unit, door ventilation). From eleven weeks of age (±25 kg) onwards until slaughter age at 29 weeks, which will be referred to as finishing period (FP), the trial pigs were present in the finishing unit. This unit comprised eight compartments located in the same barn. A minimum of two days empty period was applied before stocking new pigs in the finishing unit. All compartments were equipped with an automated temperature-regulated ventilation (door ventilation). Pelleted feed and drinking water were provided to the pigs *ad libitum*. Additional information regarding the farm can be consulted in the supplementary data section at the end of the manuscript.

### Study design

The trial started in August – September 2011 (replicate 1, R1), with the selection of 501 pigs (eleven weeks of age). The pigs originated from two consecutive groups of sows, resulting in two groups of pigs. The pigs of the first group were one month older than those of the second group. Within each group, the pigs were randomly assigned using the Excel RAND function (Excel 2010, Microsoft Corp.) to four compartments: two fully slatted compartments (0.65 $m^2$/pig) and two partially slatted floor compartments (0.64 $m^2$/pig and 0.66 $m^2$/pig). In December 2011 – January 2012, a second replicate (R2), comprising 594 pigs, was investigated. Each replicate of the study comprised thereby eight compartments (two groups of four compartments). The study was conducted after approval of the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University (approval number: EC2011/142).

### Explanatory variables: PM10 and $NH_3$

Particulate matter ($PM_{10}$) (μg/$m^3$) and $NH_3$ (ppm) inside the pig facilities were measured. A schedule of the different compartments can be found in the supplementary data. The $PM_{10}$ measurements were performed by two Grimm 1.109 spectrometers (Grimm Aerosol Technik GmbH & Co. KG) (one minute sampling interval) and two Graywolf Particle Counters-Handheld 3016IAQ (GrayWolf Sensing Solutions) (15 minute sampling interval) during the FP. The data were obtained semi-continuously by rotation of the devices twice per week per compartment and hourly averages were calculated. Bi-weekly side-by-side measurements in a random chosen compartment were performed for 30 minutes to ensure the equivalence of the four PM monitors. When differences exceeding 10%, the results of the aberrant monitor were corrected based on the side-by-side measurements (Ulens et al., 2014). Both devices were calibrated by the manufacturer.

Indoor concentrations of $NH_3$ (ppm) were measured in all eight compartments sequentially during FP with an Innova photoacoustic gas monitor 1314 (Innova AirTech Instruments) as described by Ulens et al. (2014). One hour was needed to measure all compartments sequentially. Calibration was done by the Dutch Metrology Institute VSL according to ISO/IEC 17025.

Sampling of both $PM_{10}$ and $NH_3$ was performed in the central pen of the four compartments at 0.8 m height and according to the rotation scheme for $PM_{10}$ and in each compartment for $NH_3$. The average $PM_{10}$ ($avPM_{10}$) and average $NH_3$ ($avNH_3$) concentrations were calculated for each compartment in each replicate from eleven weeks until either the day of sampling/weighing (28 weeks) of the pigs or day of slaughter (29 weeks), further referred as FP. The same calculations were considered from halfway the FP (19 weeks) onwards until the day of sampling/weighing (28 weeks) or day of slaughter (29 weeks), further referred as the second half of the FP for each replicate.

## Outcome variables

The average daily weight gain (ADG: g/pig/day) and mortality were measured. The pigs were weighed individually at eleven and 28 weeks to determine the ADG (Del Pozo Sacristán et al., 2014). Dead pigs were included in the calculation of the ADG by registering the end weight and date of mortality during the FP.

The lungs were individually examined at slaughter (29 weeks of age) in a blinded manner. First, the presence of suppurative bronchopneumonia, further referred to as pneumonia was recorded (prevalence of pneumonia), defined as macroscopical greyish to purplish consolidated areas. These lesions are generally located on the cranio-ventral parts of the lung lobes, and are demarcated from normal tissue by a sharp line following the interlobular septa (Van Alstine, 2012). Subsequently, the total area of lung tissue affected by pneumonia lesions (further referred to by extent of pneumonia lesions) representing the severity of these lesions was calculated (Morrison et al., 1985). Finally, the lungs were checked for the presence or absence of fissures and pleurisy. Fissures were defined as areas of collapsed alveoli adjoining alveolar emphysema (recovery lesions) (Kobish et al., 1993), while pleurisy was defined as fibrotic adherences between the parietal and visceral membranes of the pleural cavity (Del Pozo Sacristán et al., 2014).

To determine the presence of *M. hyopneumoniae*, ten pigs per compartment were randomly selected at the start of the trial. From these animals, nasal swabs (sterile aluminum applicator rayon tipped, COPAN) were collected at 28 weeks. The DNA-extractions for *M. hyopneumoniae* and the two steps polymerase chain reactions were performed as previously described (Stärk, 1999).

## Data and statistical analyses

Prior to statistical analysis, the $PM_{10}$ and $NH_3$ data were explored and checked for irrelevant values. These could occur because of instrument failure, repositioning of the equipment, or entering the compartment by farmer or researchers. These outlier data were discarded. A logarithmic transformation of the explanatory variables $avPM_{10}$ and $avNH_3$ was performed in order to normalize the data, further referred to as $PM_{10}$ and $NH_3$. The $PM_{10}$ and $NH_3$ concentrations were associated with the production parameters (ADG and mortality) during FP, and with

the respiratory health parameters (prevalence of pneumonia and extent of pneumonia lesions, prevalence of fissures, pleurisy and nPCR results) during the FP and the second half of the FP.

$PM_{10}$ and $NH_3$ were the explanatory variables in the statistical analyses. Seven parameters were considered as outcome variables, namely: ADG, mortality, likelihood and extent of pneumonia lesions, likelihood of fissures and pleurisy and likelihood of nPCR positivity. Associations were considered significant when $P < 0.05$.

Linear mixed regression models [MLwiN 2.26 (Rasbash et al., 2012)] were used to assess the influence of $PM_{10}$ and $NH_3$ on ADG and extent of pneumonia lesions, respectively. First, the influence of $PM_{10}$ and $NH_3$ was tested in four models in a univariable way. The assumptions of normality and homogeneity of variance of the final model were tested by examining normal probability plots of residuals and plots of residuals versus predicted values. No patterns indicating heteroscedasticity were present.

The multilevel linear statistical model may be represented mathematically as:

$Y_{ij} = \beta_0 + \beta_1 \log_{10}NH_3 + \beta_2 \log_{10}PM10 + \beta_3 Replicate + \mu_{compartment(j)} + \varepsilon_{ij}$, where $Y_{ij}$ is the continuous outcome variable (ADG or extent of pneumonia lesions), βs are the model coefficients, $\log_{10}NH_3$ is the log-transformed $NH_3$ concentration, $\log_{10}$ $PM_{10}$ is the log-transformed $PM_{10}$ concentration, replicate is the finishing period in which the pigs participated in the study, and started for replicate 1 in August - September 2011 and for replicate 2 in December 2011 - January 2012, $\mu_{compartment(j)}$ is the random effect of the compartment *i* (*i* = 1 to 8), j refers to the jth pig in the ith compartment and $\varepsilon_{ij}$ is the random error term, assumed to be normally distributed with mean 0 and variance σ2. The influence of $PM_{10}$ and $NH_3$ on mortality, the likelihood of the different lung lesions and nPCR-positivity, were analyzed by means of logistic mixed regression model using 1st order marginal quasi-likelihood algorithms (MLwiN 2.26 - Centre for Multilevel Modeling, Bristol, UK (Rasbash et al., 2012). First, $PM_{10}$ and $NH_3$ were tested in a univariable way. The fit of the models was evaluated by inspection of the pig standardized residuals plotted against the normal scores and the pig level predicted values. The Hosmer Lemeshow goodness-of-fit measure was calculated for the explanatory variable models using SAS 9.3 (PROC LOGISTIC, SAS Institute Inc., NC, USA). The results were represented as odds ratio (OR) with the 95% confidence interval calculated around these odds ratios.

The multilevel logistic statistical model may be represented mathematically as:

$(g)Y_{ij} = \beta_0 + \beta_1 \log_{10}NH_3 + \beta_2 \log_{10}PM_{10} + \beta_3 Replicate + \mu_{compartment(j)} + \varepsilon_{ij}$, where (g) refers to the logit link function, $Y_{ij}$ is the probability of the outcome variable on the logit scale (lung lesions and nPCR results), βs are the model coefficients, $\log NH_3$ is the log-transformed $NH_3$ concentration, $\log_{10}PM_{10}$ is the log-transformed $PM_{10}$ concentration, replicate is the finishing period in which the pigs participated in the study, and started for replicate 1 in August - September 2011 and for replicate 2 in December 2011- January 2012, $\mu_{compartment(j)}$ is the random effect of the compartment *i* (*i* = 1 to 8), j refers to the jth pig in the ith compartment and $\varepsilon_{ij}$ is the random error term, assumed to be normally distributed with mean 0 and variance σ2. In total, 20 separate univariable models were tested *i.e.* four predictor variables ($PM_{10}$, $NH_3$, $PM_{10}$ during the second half of the FP and $NH_3$ during the second half of the FP) and five different outcome variables. In case both $PM_{10}$ and $NH_3$ were significantly associated with the specific outcome variables, they were simultaneously included in a multivariable model (Table 5). In all models, replicate was included as predictor variable to account for seasonal

influence and compartment was included as random effect to correct for clustering of pigs within a compartment.

## Results

Average values of the explanatory variables and outcome variables in each replicate of the study are shown in Tables 1 and 2, and 3, respectively. Results of the univariable and multivariable models are shown in Table 4 and 5, respectively. Neither $PM_{10}$ FP nor $NH_3$ FP were significantly associated with ADG and mortality (Table 4).

The $PM_{10}$ second half of the FP concentration was positively and significantly associated with the likelihood of pneumonia (OR=8.72; P=0.015) (Table 4).

A positive significant association was found between $PM_{10}$ and the extent of pneumonia lesions (FP; P=0.04 and second half of the FP; P<0.009) (Table 4).

A significant positive association was found between $PM_{10}$ FP and $NH_3$ FP with the odds of prevalence of pleurisy (OR=20.91; P<0.001 and OR=21.54; P=0.003) (Table 4). In the multivariable model, a trend of a positive association between $NH_3$ FP and the likelihood of pleurisy was observed, while $PM_{10}$ FP remained significantly associated with the likelihood of pleurisy (OR=8.85; P=0.049) (table 5). Increasing $PM_{10}$ (second half of the FP) increased (OR=40.85; P<0.001) the likelihood of pleurisy (Table 4).

A significant positive association was found between $PM_{10}$ FP (OR=328.00; P=0.01) and $NH_3$ FP (OR=70.37; P=0.049) and the likelihood of nPCR-positivity (Table 4). However, in the multivariable model, only a trend of a positive association between both $PM_{10}$ and $NH_3$ and the likelihood of nPCR-positivity remained (Table 5). Similar results were observed for the association between the likelihood of nPCR-positivity and the $PM_{10}$ and $NH_3$ during the second half of the FP (Table 4 and 5).

**Table 1:** Averages of particulate matter 10 ($PM_{10}$) concentration and the outcome variables average daily gain (ADG), nested polymerase chain reaction (nPCR) results to detect *M. hyopneumoniae* DNA, mortality, and different lung lesions detected at slaughter (extent of pneumonia, prevalence of pneumonia, fissures and pleurisy). The given data are the average values of the $PM_{10}$ data used for the associations in the univariable and/or multivariable models for each outcome variable. The different start and end time measuring time point are taken into account for each outcome variable and can be seen between brackets after each outcome variable.

| Outcome variable (period) | Explanatory variable | | |
|---|---|---|---|
| | Replicate | Average $PM_{10}$ ± SD (µg/m³) | Minimum-maximum $PM_{10}$ (µg/m³) |
| **ADG (11-28 wk)** | 1 | 2210 ± 558 | 1691 – 3280 |
| | 2 | 2263 ± 392 | 1820 – 2817 |
| | 1+2 | 2239 ± 476 | 1691 – 3280 |
| **Mortality and Lung lesions (11-29 wk)** | 1 | 2230 ± 548 | 1707 – 3280 |
| | 2 | 2231 ± 424 | 1723 – 2817 |
| | 1+2 | 2230 ± 485 | 1707 – 3280 |
| **Lung lesions (19-29 wk)** | 1 | 3030 ± 660 | 2204 – 4401 |
| | 2 | 2229 ± 511 | 1667 – 2985 |
| | 1+2 | 2596 ± 707 | 1667 – 4401 |
| **nPCR (11-28 wk)** | 1 | 2254 ± 578 | 1691 – 3280 |
| | 2 | 2236 ± 387 | 1820 – 2817 |
| | 1+2 | 2245 ± 486 | 1691 – 3280 |
| **nPCR (19-28 wk)** | 1 | 3063 ± 709 | 2240 – 4412 |
| | 2 | 2210 ± 491 | 1667 – 2985 |
| | 1+2 | 2620 ± 740 | 1667 – 4412 |

wk: age of the pigs in weeks
Period: average PM10 value calculated for replicate 1 and 2 and for both replicates combined for FP from the beginning of the trial (11 wk)- until final weighing date/sampling date (28 wk) or until slaughter age (29 wk) or second half of the FP: average PM10 value calculated over the second half of the finishing period 1, 2 or 1+2: from halfway the finishing period (19 wk) until slaughter age (29 wk)
ADG: average daily weight gain, unit g/pig/day
SD: standard deviation

**Table 2:** Averages of $NH_3$ concentrations (ppm) calculated over the entire finishing period and for the second half of the finishing period and the outcome variables average daily gain (ADG), nested polymerase chain reaction (nPCR) results to detect M. hyopneumoniae DNA, mortality, and different lung lesions detected at slaughter (extent of pneumonia, prevalence of pneumonia, fissures and pleurisy). The given data are the average values of the $NH_3$ data used for the associations in the univariable and/or multivariable models for each outcome variable. The different start and end time measuring time point are taken into account for each outcome variable and can be seen between brackets after each outcome variable.

| Outcome variable (period) | Explanatory variable | | |
|---|---|---|---|
| | **Replicate** | **Average $NH_3$ ± SD (ppm)** | **Minimum-maximum $NH_3$ (ppm)** |
| **ADG (11-28 wk)** | 1 | 27.1 ± 7.9 | 15.5 - 37.4 |
| | 2 | 27.1 ± 3.7 | 22.3 - 33.0 |
| | 1+2 | 27.1 ± 6.0 | 15.5 - 37.4 |
| **Mortality & Lung lesions (11-29 wk)** | 1 | 29.8 ± 6.6 | 17.2 - 38.2 |
| | 2 | 26.9 ± 3.7 | 22.1 - 32.8 |
| | 1+2 | 28.2 ± 5.4 | 17.2 - 38.2 |
| **Lung lesions (19-29 wk)** | 1 | 33.9 ± 4.7 | 26.2 - 41.0 |
| | 2 | 18.4 ± 2.8 | 15.3 - 22.8 |
| | 1+2 | 25.5 ± 8.6 | 15.3 - 41.0 |
| **nPCR (11-28 wk)** | 1 | 27.2 ± 8.0 | 15.5 - 37.4 |
| | 2 | 27.3 ± 3.8 | 22.3 - 33.0 |
| | 1+2 | 27.3 ± 6.2 | 15.5 - 37.4 |
| **nPCR (19-28 wk)** | 1 | 32.7 ± 5.3 | 25.8 - 40.1 |
| | 2 | 18.6 ± 2.8 | 15.1 - 22.8 |
| | 1+2 | 25.3 ± 8.2 | 15.1 - 40.1 |

Period: average $NH_3$ value calculated for replicate 1 and 2 and for both replicates combined for FP: from the beginning of the trial (11 wk) - until final weighing date/sampling date (28 wk) or until slaughter age (29 wk) or second half of the FP: average $NH_3$ value calculated over the second half of the finishing period 1, 2 or 1+2: from halfway the finishing period (19wwk) until slaughter age (29 wk)
ADG: average daily weight gain, unit g/pig/day
wk: age of the pigs in weeks
SD: standard deviation
ppm: parts per million

**Table 3:** Descriptive statistics of the outcome variables: ADG±SD, mortality, prevalence of pneumonia lesions, extent of pneumonia lesions ±SD and prevalence of fissures and pleurisy and nPCR to detect *M. hyopneumoniae* DNA results in replicate 1, replicate 2 and calculated for both replicates. Age (in weeks) points out over which period (ADG, mortality) or at which timepoint (prevalence of pneumonia, extent of pneumonia lesions, prevalence of fissures and pleurisy and nested polymerase chain reaction (nPCR) to detect *M. hyopneumoniae* DNA) the outcome variable was determined.

| Outcome variables | Age (weeks) | Replicate 1 (n = 501[a]) | Replicate 2 (n = 594[a]) | Replicate 1+ 2 (n = 1095[a]) |
|---|---|---|---|---|
| **ADG (g/pig/day)** | 11-28 | 719 ± 88 [711-727] | 676 ± 132 [665-687] | 695 ± 116 [688-702] |
| **Mortality (%)** | 11-29 | 5.39 (27 / 501) | 2.53 (15 / 594) | 3.84 (42 / 1095) |
| | | n = 289[b] | n = 481[b] | n = 770[b] |
| **Prevalence of pneumonia (%)** | 29 | 52.2 | 69.4 | 63.0 |
| **Extent of pneumonia lesions (%)** | 29 | 8.68 ± 11.9 [7.30-10.06] | 7.99 ± 11.4 [6.97-9.01] | 8.25 ± 11.6 [7.43-9.07] |
| **Prevalence of fissures (%)** | 29 | 33.6 | 35.6 | 34.8 |
| **Prevalence of pleurisy (%)** | 29 | 54.1 | 69.5 | 63.8 |
| **nPCR (%)** | 28 | 38.5 (25 / 65) | 18.4 (14 / 76) | 27.7 (39 / 141) |

ADG: average daily gain

g: gram

a=number of animals

b=number of lungs investigated in the slaughter house

[lower level confidence interval-upper level confidence interval]

**Table 4:** Results of univariable models between both the log10-transformed average $PM_{10}$ and log10-transformed average $NH_3$ calculated over the entire finishing period (11-28/29 weeks) and/or over the second half of the finishing period (19 – 28/29 weeks) on the different outcome variables: average daily weight gain (ADG), the likelihood of mortality, extent of pneumonia lesions, the likelihood of pneumonia lesions, fissures and pleurisy and the likelihood of nested polymerase chain reaction (nPCR) positivity to detect *M. hyopneumoniae* DNA.

| Outcome variables | Explanatory variables | $\beta_0$ | $\beta_1$ | SE | OR | CI | P-value |
|---|---|---|---|---|---|---|---|
| **ADG** | **$PM_{10}$ FP** | 0.98 | -0.08 | 0.04 | - | - | 0.08 |
| | **$NH_3$ FP** | 0.82 | -0.07 | 0.04 | - | - | 0.08 |
| **Likelihood of Mortality** | **$PM_{10}$ FP** | 4.44 | -2.23 | 1.92 | 0.11 | 0.00-4.62 | 0.245 |
| | **$NH_3$ FP** | 0.52 | -2.26 | 1.98 | 0.10 | 0.00-5.10 | 0.255 |
| **Likelihood of pneumonia** | **$PM_{10}$ FP** | -3.30 | 1.03 | 0.87 | 2.80 | 0.51-15.43 | 0.236 |
| | **$NH_3$ FP** | -1.32 | 0.94 | 1.06 | 2.56 | 0.32-20.26 | 0.372 |
| | **$PM_{10}$ $2^{nd}$FP** | -7.50 | 2.17 | 0.89 | 8.72 | 1.52-49.92 | 0.015* |
| | **$NH_3$ $2^{nd}$FP** | 1.02 | -0.61 | 1.42 | 0.54 | 0.03-8.87 | 0.669 |
| **Extent of pneumonia lesions** | **$PM_{10}$ FP** | -0.26 | 0.10 | 0.05 | - | - | 0.04* |
| | **$NH_3$ FP** | -0.07 | 0.11 | 0.06 | - | - | 0.08 |
| | **$PM_{10}$ $2^{nd}$FP** | -0.39 | 0.14 | 0.05 | - | - | 0.009* |
| | **$NH_3$ $2^{nd}$ FP** | -0.06 | 0.091 | 0.09 | - | - | 0.299 |
| **Likelihood of fissures** | **$PM_{10}$ FP** | 0.99 | -0.53 | 0.91 | 0.59 | 0.10-3.50 | 0.562 |
| | **$NH_3$ FP** | -0.04 | -0.48 | 1.10 | 0.62 | 0.07-5.37 | 0.665 |
| | **$PM_{10}$ $2^{nd}$FP** | 1.26 | -0.57 | 0.94 | 0.57 | 0.09-3.57 | 0.544 |
| | **$NH_3$$2^{nd}$ FP** | 0.86 | -1.04 | 1.53 | 0.35 | 0.02-7.02 | 0.495 |
| **Likelihood of pleurisy** | **$PM_{10}$ FP** | -9.78 | 3.04 | 0.89 | 20.91 | 3.65-119.63 | < 0.001* |
| | **$NH_3$FP** | -4.40 | 3.07 | 1.05 | 21.54 | 2.75-168.68 | 0.003* |
| | **$PM_{10}$ $2^{nd}$FP** | -12.80 | 3.71 | 0.94 | 40.85 | 6.47-257.86 | < 0.001* |
| | **$NH_3$ $2^{nd}$FP** | -3.79 | 2.56 | 1.38 | 12.94 | 0.87-193.41 | 0.064 |
| **Likelihood of nPCR positive samples** | **$PM_{10}$ FP** | -19.89 | 5.79 | 2.34 | 328.00 | 3.34-32189.64 | 0.01* |
| | **$NH_3$ FP** | -6.56 | 4.25 | 2.16 | 70.39 | 1.02-4882.83 | 0.049* |
| | **$PM_{10}$ $2^{nd}$FP** | -18.66 | 5.22 | 2.31 | 185.49 | 1.99-17265.75 | 0.02* |
| | **$NH_3$ $2^{nd}$FP** | -14.14 | 9.02 | 3.51 | 8275.05 | 8.60-7966852.07 | 0.01* |

Each row per outcome variable represents one univariable model
ADG: average daily weight gain
$PM_{10}$/$NH_3$ FP: log10-transformed average value of $PM_{10}$ or $NH_3$ calculated over the entire finishing period: 11-29 weeks of age
$PM_{10}$/$NH_3$ $2^{nd}$ FP: log10-transformed average value of $PM_{10}$ or $NH_3$ calculated over the second half of the finishing period: 19-29 weeks
$\beta_0$: Intercept of the linear/logistic regression equitation
$\beta_1$: regression coefficient of the linear/logistic regression equitation
SE: standard error
OR: odds ratio = exponent ($\beta_1$)
CI: confidence interval
P-values with an asterisk (*) are significant (P<0.05)
-: OR in continuous variables were not applicable

**Table 5**: Multivariable associations of the explanatory variable log10 transformed average $PM_{10}$ and log10 transformed average $NH_3$ calculated over the entire finishing period (11-28/29 weeks) or/and over the second half of the finishing period (19 – 28/29 weeks) associated with the outcome variable likelihood of pleurisy and with likelihood nested polymerase chain reaction (nPCR) positivity to detect *M. hyopneumoniae* DNA.

| Outcome variable | Explanatory variable | $\beta_0$ | $\beta_1$ | SE | OR | CI | P-value |
|---|---|---|---|---|---|---|---|
| **Likelihood of pleurisy** | **$PM_{10}$ FP** | -9.29 | 2.18 | 1.11 | 8.85 | 1.00-77.91 | 0.049* |
| | **$NH_3$ FP** | | 1.56 | 1.33 | 4.76 | 0.35-64.51 | 0.239 |
| **nPCR** | **$PM_{10}$ FP** | -19.230 | 5.01 | 2.95 | 149.46 | 0.46-48484.53 | 0.09 |
| | **$NH_3$ FP** | | 1.44 | 2.58 | 4.20 | 0.03-656.50 | 0.58 |
| **nPCR** | **$PM_{10}$ 2ndFP** | -21.440 | 3.18 | 2.77 | 24.14 | 0.11-5503.83 | 0.25 |
| | **$NH_3$ 2ndFP** | | 6.52 | 3.90 | 680.62 | 0.32-1426764.71 | 0.09 |

Each row per outcome variable represents one multivariable model
$PM_{10}/NH_3$ FP: log10-transformed average value of $PM_{10}$ or $NH_3$ calculated over the entire finishing period: 11-29 weeks of age
$PM_{10}/NH_3$ 2ndFP: log10-transformed average value of $PM_{10}$ or $NH_3$ calculated over the second half of the finishing period: 19-29 weeks
$\beta_0$: Intercept of the logistic regression equitation
$\beta_1$: regression coefficient of the logistic regression equitation
SE: standard error
OR: odds ratio = exp ($\beta_1$)
CI: confidence interval
P-values with an asterisk (*) are significant ($P<0.05$)

## Discussion

The present study investigated the influence of $PM_{10}$ and $NH_3$ on performance, lung lesions and the occurrence of *M. hyopneumoniae* in grower-finishing pigs with clinical problems of EP. The remaining finding of the multivariable models was that the $PM_{10}$ concentration significantly increased the odds of pleurisy (FP). Other statistically significant findings for both $PM_{10}$ and $NH_3$ (nPCR FP and second half of the FP) in the multivariable models were reduced to non-significance. Particulate matter 10 appeared to have a greater impact than $NH_3$ on the respiratory parameters in the univariable models prevalence of pneumonia and pleurisy (second half of the FP) and extent of pneumonia (FP and second half FP).

As the study was conducted in one commercial herd with clinical problems of enzootic pneumonia in grower-finishers, the results cannot be generalized as such to all pig herds, but only to similar farms. However, the housing, feeding and management practices were similar to many pig farms in Belgium and Western Europe. To see any effect of $PM_{10}$ and $NH_3$ on presence of lung lesions caused by *M. hyopneumoniae*, it was key that a farm with clinical problems of *M. hyopneumoniae* and with a sufficiently high level of $PM_{10}$ and $NH_3$ concentrations was selected. One of the compartments in the farm had a total unobstructed floor area of 0.64 $m^2$/pig per pen, while an area of 0.65 $m^2$/pig is required for pigs from 85 kgs until 110 kgs as stated in article 3 of council directive 2008/120/EC (European Council, 2008). From 110 kgs onwards the floor space area per pig is required to be 1.00 $m^2$/pig. Likewise the average weight at the end of the finishing stage in most pens exceeded 110 kgs. In practice, temporary overstocking at the end of the finishing period (when the allowed stocking density shifts from 0.65 to 1 $m^2$ per pig i.e. at 110 kg is quite common, although it is in contrast with the European law. It is well known that overstocking contributes to respiratory problems (Stärk, 2000). These problems addressed above can be the reason why *M. hyopneumoniae* was still causing fairly large clinical problems in the farm, despite vaccination of the piglets. To reduce the transmission pressure and $PM_{10}$ and $NH_3$ concentration it would be recommended to comply with the stocking density as stated in the European regulation. Overstocking might be a potential confounder in this study, however including compartment into the model as a random effect takes into account the association between observations within the same compartment and thus controls for any potential confounding factor at compartment level.

No recommendations in Belgian legislation for animals regarding airborne dust and $NH_3$ are given. However, the maxima of the average $PM_{10}$ concentrations measured in this farm were higher than the upper level of the range given in Van Ransbeeck et al. (2013b) (2288 $\mu g/m^3$). Following threshold for $NH_3$ regarding swine health is suggested based on a dose-response correlation to swine health: eleven ppm (Donham, 1990). The $NH_3$ values in this farm showed higher levels than this recommended value.

A simultaneous, long term and detailed measurement of environmental parameters, $PM_{10}$ and $NH_3$ was performed to relate these parameters to performance as well as to respiratory parameters. No other climatological data such as temperature, rainfall or wind were included in the analysis. $PM_{10}$ and $NH_3$ were measured during the entire year, thereby correcting for seasonal influence. Humidity and ventilation can influence the $PM_{10}$ and $NH_3$ levels as well. However by performing the semi-continuous ($PM_{10}$) or continuous ($NH_3$) measurement during an entire day and night, during four different seasons, these factors were taken into

account. Inter-compartment variation was taken into consideration by including replicate and compartment respectively into the model. The study was conducted in one farm. This has the advantage that the main influencing factors as management, husbandry activity, animal activity, age of the animals, feeding system and type of feed (Dawson, 1990; Harry, 1978; Pedersen et al., 2000) were the same for all fattening pigs included in the study and it is therefore not necessary to include these factors into the statistical model. Adding a control group with low $PM_{10}$ and $NH_3$ concentrations in the same farm would have had practical difficulties: which technique to use, more equipment to maintain and to check, etc. Moreover the main interest was to investigate associations between $PM_{10}$ and $NH_3$ on the one hand and production parameters and lung lesions on the other hand. From that perspective, and to make the study not unnecessary complicated, the authors opted not to add a control group. Between different compartments in one farm there are certain factors which can diminish the $PM_{10}$ and/or $NH_3$ levels: lowering the level of light and thus animal activity in the stable, not using litter and dry feed, reduction of the slurry pit surface (sloped pit walls), floor material (iron, metal, plastic versus concrete slats), a good drainage of the faeces through the floor (Dawson, 1990; Pearson and Sharples, 1995; Philippe et al., 2011). High ventilation rates and low temperatures promote the production of particulates (Kim et al., 2005; Stärk, 1999). However these listed factors were not measured as this fell out of the scope of this study. Temporal variation of the $PM_{10}$ and $NH_3$ data was explored (data not shown). The $PM_{10}$ levels were, according to the rotation scheme of the devices, measured with a one minute interval or 15 minute interval during day and night. In this way higher PM concentrations which occur during the day were taken into account as well (Takai et al., 1998). According to Van Ransbeeck et al. (2012), the variation in location of the devices by the rotation scheme can be neglected compared to the importance of measurements carried out during many stages of the whole finishing period (Van Ransbeeck et al., 2012).

A chronic effect of $PM_{10}$ and $NH_3$ on the outcome variables was emphasized as long term measurements (entire finishing period) were performed. However, also the maximum concentration might have influenced the outcome variables, as at one time point inhaling a high concentration of hazardous substances and micro-organisms can have a detrimental and permanent effect on the pig's health.

Average daily growth in the study was rather low compared to today's standards [725 g per pig per day recorded in a national technical data report (Bulens et al., 2013)]. However it should be noted that dead pigs were included in the calculation of the ADG. In this farm artificial insemination with Piétrain semen was performed. Piétrain-sired progeny accumulate less fat in comparison with other sires used, however they grow more slowly (Edwards et al., 2006; Labroue et al., 1999). Other than the type of boar being used, the poor growth rate in this farm could also be due to the clinical problems with *M. hyopneumoniae*. It is well known that enzootic pneumonia decreases performance such as ADG (Thacker and Minion, 2012). No significant association of both environmental parameters, $PM_{10}$ and $NH_3$ with ADG could be found. This finding is in accordance with the observations of (Van 't Klooster, 1993), (Jansen and Feddes, 1995) and (Takai et al., 1995), although these authors only considered particulates and not $NH_3$.

The overall mortality rate during the FP was 3.84%. No significant association was observed between the $PM_{10}$ and $NH_3$ concentration and mortality. One other study associated PM with death loss (Donham, 1991). The latter author found a positive and significant correlation between total and respirable PM and death loss in

fatteners. However no correlation with the thoracal dust fraction, which is included in the $PM_{10}$ fraction measured in our study, was made. This can be the reason why the result of Donham et al. (1991) was not in line with our findings.

The pigs were slaughtered at 29 weeks. Some pigs were not slaughtered at that time (as they did not reach sufficient weight yet), but two weeks later. The measurements of $PM_{10}$ and $NH_3$ and the associations with the outcome variables were determined from eleven weeks onwards until 29 weeks of age (and for the lung lesions and nPCR results as well from 19 – 29 weeks), as a lower number of pigs in the pens would have influenced the $PM_{10}$ and $NH_3$ concentrations and therefore the outcome of the results. A high prevalence of pneumonia lesions was recorded at slaughter (63.0%) and these active pneumonia lesions will appear at earliest one week and reach a maximum four weeks post infection and will be recovered resulting in interlobular scar retractions more or less twelve weeks after infection (Kobish et al., 1993; Sørensen et al., 1997). Thus a large part of these pigs with active pneumonia lesions at slaughter were infected maximum twelve weeks before slaughter, which is roughly half way the finishing period. This means that not only *M. hyopneumoniae* was circulating in the earlier stages of the finishing period which can be determined by the fissurae lesions, but also during the second half of the finishing stage. On the other hand the pneumonia lesions found at slaughter were not that severe (score 8.25). Del Pozo Sacristán et al. (2012), using the same scoring system, found similar prevalences (20.5, 13.1, 23.0) and extent of pneumonia lesions (15.0, 13.4 and 14.9) in three groups of slaughter pigs. Higher $PM_{10}$ concentrations were significantly associated with the prevalence of pneumonia (second half of the FP) and with the extent of pneumonia lesions (FP, second half of the FP). Higher $NH_3$ concentrations resulted in a trend of the extent of pneumonia lesions (FP and second half of the FP) being higher. The finding was in agreement with Jolie et al (1999b). They found more macroscopic lung lesions indicative for *M. hyopneumoniae* when the air of the compartment was more contaminated with PM and $NH_3$, although no significance level was mentioned (Jolie et al., 1999b). If an association between PM and $NH_3$ on the one hand and prevalence of pneumonia and extent of pneumonia lesions on the other hand can be assumed, it is more likely that this association will be expressed in the second half of the FP. The onset of the pneumonia lesions will not likely have started earlier than twelve weeks before slaughter house check as these pneumonia lesions heal after a period of twelve weeks (Sørensen et al., 1997). Based on this finding, it is clear that the indoor air quality for the finishing pig is not only important in the early stages of life as stated by Fablet (2009), but during the second half of the production stage as well (Fablet, 2009). Upon healing of pneumonia lesions (Sørensen et al., 1997), fissures develop and remain for a period of 12 weeks (Kobish et al., 1993; Van Alstine, 2012). An overall prevalence of 34.8% was detected at slaughter which indicates that the pigs had been experiencing pneumonia lesions earlier on in the FP as well, as already mentioned. To the author's knowledge, no other reports describe the association between PM and $NH_3$ on the one hand and the prevalence of fissures on the other hand. In our study no association between these parameters was detected. It was found that slaughter pigs seropositive for *M. hyopneumoniae* had chronic pleurisy in 29% of the cases (Van Alstine, 2012). The overall value detected at slaughter in our study was 63.8% which is high in comparison with the 20.76% found in Meyns et al. (2011). Particulates and ammonia (FP) were positively associated with the prevalence of pleurisy lesions, with only $PM_{10}$ remaining significantly associated with the prevalence of pleurisy in the multivariable model. Donham et al. (1991) found a significant correlation between

PM and $NH_3$ and pleurisy lesions (univariably tested) in finishing pigs as well notwithstanding the differences in experimental design compared with our study. Particulates and $NH_3$ can tip the balance between host and respiratory invaders in favor of the invaders (Donham, 1991). This study focused mainly on the influence of $PM_{10}$ and $NH_3$ on the presence of *M. hyopneumoniae*. Therefore, our interest was to associate $PM_{10}$ and $NH_3$ to the prevalence of *M. hyopneumoniae* and other diseases e.g. atrophic rhinitis were not investigated. An overall result of 27.7% of positive samples for *M. hyopneumoniae* in the nasal swabs was obtained and the overall prevalence of pneumonia lesions was 63.0%. The discrepancy between the results of the nPCR and the pneumonia prevalence can be due to several reasons. First, pneumonia lesions detected at slaughter are characteristic for *M. hyopneumoniae* infections in the farm and are frequently used to assess the severity of enzootic pneumonia in the farm. However, it is well known that the lesions are not pathognomonic. Secondly, other pathogens, especially viruses (e.g. Influenza virus) can induce similar lesions (Sibila et al., 2009). Another reason for this above mentioned discrepancy might be that only a subsample of the 1095 animals in the farm was investigated. It is also possible that due to coincidence a lower number of positive animals was detected than the animals that were really infected with *M. hyopneumoniae*, although the samples were taken at random. Nasal swabs were collected, as it is safer for the researchers and less stressful for the animals than taking tracheal swabs (Fablet et al., 2010; Thacker, 2004). However, nasal swabs are less sensitive to detect DNA of *M. hyopneumoniae* than tracheal swab or BALf. When including both $PM_{10}$ and $NH_3$ in the multivariable model, only a trend of $PM_{10}$ and $NH_3$ causing a higher number of nPCR positive results remained in FP and second half of the FP. To the author's knowledge no previous work ever associated PM or $NH_3$ with DNA of *M. hyopneumoniae* detectable in pigs.

## Conclusions

The present study demonstrated that increasing $PM_{10}$ concentrations resulted in a higher prevalence of pleurisy lesions under field conditions. When $PM_{10}$ was considered in the univariable model, it appeared to have a greater impact than $NH_3$ on the respiratory parameters, in the second half of the FP for the prevalence of pneumonia and pleurisy. This statement was also true for the extent of pneumonia lesions (FP and second half of the FP).

The effect of $PM_{10}$ and $NH_3$ on other parameters (nPCR FP and second half of FP) in the multivariable model was reduced to non-significance. This implies that more research is necessary to draw further conclusions. A higher sample size can be a solution to investigate the trends found in this study into depth, however conducting a larger study will have practical implications in terms of feasibility of the study. These findings indicate that the respiratory health of finishing pigs was significantly affected by $PM_{10}$.

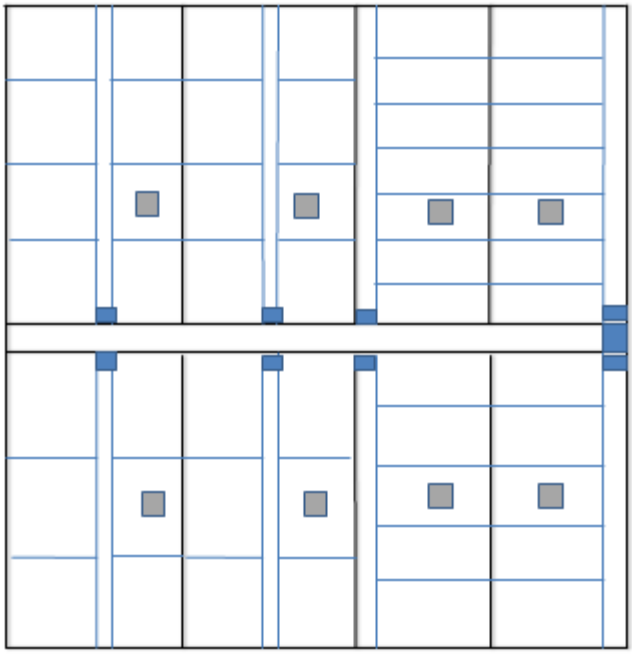
## Supplementary data files

**Table 1**: Description of the herd enrolled in the study

| | |
|---|---|
| **Number of sows** | 210 |
| **Sow breed** | JSR-hybrid |
| **Type of herd** | Closed herd |
| **Type of batch farrowing system for sows** | 4-week |
| **Vaccination gilts and sows against *Mycoplasma hyopneumoniae*** | No |
| **Vaccination of gilts and sows against other agents** | SIV (Suvaxyn Flu®, Zoetis)<br>PRRSV (Porcilis PRRS®, MSD)<br>*E. rhusiopathiae* + Parvovirus (Parvoruvax®, Merial)<br>*E. coli* (Neocolipor®, Merial)<br>PCV-2 (Porcilis PCV2®, MSD)<br>AR (Porcilis AR-T DF®, MSD) |
| **Number of piglets weaned per sow per year** | 27 |
| **Management of suckling piglets** | Day 0 (birth day): teeth clipping, umbilicus disinfection, ceftiofur (Naxcel®, Zoetis)<br>Day 3: Fe, IM (Ferrohipra®, Laboratorius Hipra), surgically neutering of the males, tail docking, ceftiofur (Naxcel®, Zoetis) |
| **Vaccination piglets against *Mycoplasma hyopneumoniae*** | Day 3 + Day 21: Suvaxyn Mhyo® (Zoetis) |
| **Vaccination piglets against other agents** | Day 21: PCV-2, (Porcilis® PCV, MSD) |
| **Strategic medication of nursery piglets** | Amoxycillin and colistin (Amoxycol WSP®, Dopharma), in drinking water, two times five days administration and five amoxicillin and colistin-free days in between |
| **Strategic medication finishing pigs** | No |
| **Deworming finishing pigs** | Levamisole hydrochloride 80%® (Kela Laboratoria N.V.), in drinking water, every 2 months |

JSR: John Sykes Rymer Genetics Limited
SIV: Swine Influenza virus
PRRSV: Porcine Reproductive and Respiratory Syndrome Virus
*E. rhusiopathiae*: *Erysipelothrix rhusiopathiae*
*E. coli*: *Escherichia coli*
PCV-2: Porcine Circovirus type 2
AR: Atrophic rhinitis

**Fig. 1: Schematic overview of the pig compartments included in the study.**
Compartments are separated with a black line, the corridors are marked with a blue line. The main corridor is marked with two black lines. Compartments are numbered from one to eight, in a counter clockwise direction, starting with compartment one (left, below). Doors are marked with a blue square. The cage with the equipment to measure the $PM_{10}$ and $NH_3$ data are marked with a grey square.



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# *3.3. Clinical impact of a simultaneous in-feed administration of deoxynivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol on the severity of an experimental Mycoplasma hyopneumoniae infection in pigs*

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## Abstract

The mycotoxin deoxynivalenol (DON) is highly prevalent in cereals in moderate climates and therefore pigs are often exposed to a DON-contaminated diet. Pigs are highly susceptible to DON and intake of DON-contaminated feed may lead to an altered immune response and may influence the pathogenesis of specific bacterial diseases. Therefore, the maximum guidance level in feed is lowest in this species and has been set at 900 micrograms (µg)/kilogram (kg) feed by the European Commission. This study aimed to determine the effect of in-feed administration of a moderately high DON concentration (1,514 µg/kg) on the severity of an experimental *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) infection in weaned piglets. Fifty *M. hyopneumoniae*-free piglets were assigned at 30 days of age [study day (D)0] to four different groups: 1) negative control group (NCG; n=5), 2) DON-contaminated group (DON; n=15), 3) DON-contaminated and *M. hyopneumoniae*-inoculated group (DONMHYO; n=15), 4) *M. hyopneumoniae*-inoculated group (MHYO; n=15). The piglets were fed the experimental diets *ad libitum* for five weeks and were monitored during this period and euthanized at day 35 [27 days post infection (DPI)] or 36 (28 DPI). The main parameters under investigation were macroscopic lung lesions (MLL) at euthanasia, respiratory disease score (RDS) from day 8 until day 35, histopathologic lesions and log copies of *M. hyopneumoniae* DNA detected by qPCR, determined at the day of euthanasia.

No significant difference was obtained for MLL at euthanasia, RDS (8-35), histopathologic lung lesions and log copies of *M. hyopneumoniae* DNA in the DONMHYO and MHYO group and consequently, no enhancement of the severity of the *M. hypneumoniae* infection could be detected in the DONMHYO compared to the MHYO group.

Under present conditions, the findings imply that feed contaminated with DON (1,514 µg/kg) provided to weaned pigs for five weeks did not increase the severity of an experimental *M. hyopneumoniae* infection. Further research is needed to investigate the impact of DON on *M. hyopneumoniae*-infections in a multi-mycotoxin and multi-pathogen environment.

## Introduction

The mycotoxin deoxynivalenol (DON) is a fungal metabolite produced mainly by *Fusarium graminearum* and *Fusarium culmorum* (Bracarense et al., 2012; Whitlow et al., 2010). *Fusarium* species-produced mycotoxins, of which by toxicological viewpoint the trichothecenes DON and T-2 toxin, zearalenone and fumonisins are the most important (Antonissen et al., 2014a), have been reported worldwide in many cereal-based cropping systems. *Fusarium* species have traditionally been associated with temperate cereals, as these fungi require lower temperatures for growth and mycotoxin production (Placinta et al., 1999). Indeed, DON is one of the most common natural mycotoxin contaminants of wheat and other small cereal grains harvested in moderate climate zones (Kostelanska et al., 2011; Miller, 2008; Rasmussen et al., 2012). Extensive data on global mycotoxin occurrence showed that 59% of 5,819 samples of animal feed tested positive on DON presence (Antonissen et al., 2014a; Placinta et al., 1999; Rodrigues and Naehrer, 2012). In low doses, DON causes anorexia, decreased weight gain and immune stimulation (Bracarense et al., 2012). Pigs are known to be a very sensitive animal species to DON, mainly because of the high oral bioavailability and differences in metabolism of this mycotoxin compared to other species (Bracarense et al., 2012). In moderate to high doses (above 840 μg/kg feed), decreased feed intake or feed refusal, vomiting and immune suppression are seen (Accensi et al., 2006; Bracarense et al., 2012; Dersjant-Li et al., 2003). In fact, the European Union (EU) recommended maximum pig feed guidance level for DON is 900 μg/kg, which is the lowest one compared to other farm animal species for total diets and compounds of total diets. For adult ruminants and poultry a maximum of 5,000 μg/kg and 2,000 μg/kg for calves and lambs are set as guidance levels (Commission recommendation of 17 August, 2006). On top of that, the pig consumes a cereal rich diet and DON is frequently detected in wheat, barley, corn and by-products (Accensi et al., 2006; Pierron et al., 2016).

It is known that DON can have an impact on the pathogenesis of several bacterial diseases (Antonissen et al., 2015; Antonissen et al., 2014a; Antonissen et al., 2014b). Exposing porcine ileal loop tissue to a DON-concentration of 1 μg/ml, potentiated the inflammatory response and significantly enhanced *Salmonella* Typhimurium invasion in and passage of the bacterium across the intestinal epithelium (Vandenbroucke et al., 2011). Furthermore, DON (0.025 μg/ml) induced an enhanced uptake of *Salmonella* Typhimurium in porcine macrophages, indicating the capacity of DON to modulate the innate immune system, and thus to increase the susceptibility of the pig to *Salmonella* Typhimurium infections (Vandenbroucke et al., 2009). Deoxynivalenol might, due to the immunomodulatory effect on the host and/or the immediate impact on the pathogen, have an impact on the course of respiratory infectious diseases in swine. However only limited *in vivo* information is available. A three-week ingestion period of feed contaminated with high levels of 2,500 μg/kg and 3,500 μg/kg DON resulted in a higher viremia and lung viral load in case of a Porcine Circovirus type 2 (PCV2) infection, and a lower body weight gain, more lung lesions and mortality in porcine reproductive and respiratory syndrome virus (PRRSv)-infected pigs, respectively (Savard et al., 2014; Savard et al., 2015b). Pigs receiving fumonisin B1 in a concentration of 10,000 μg/kg feed, and dually infected with *Bordetella bronchiseptica* (*B. bronchiseptica*) and *Pasteurella multocida*, (*P. multocida* ) were at greater risk to develop pneumonia and had an increase of the extent and severity of the pathological changes compared to dually infected pigs that did not receive fumonisin B1 (Pósa et

al., 2011). Oral gavage of *P. multocida*-infected pigs with a crude extract of fumonisin B1 in a concentration of 500 μg/kg body weight (BW) per day for a period of seven days resulted in the pigs coughing more, in increased bronchoalveolar lavage fluid total cells, macrophages and lymphocytes, and resulted in an increased occurrence of lung lesions compared to the pigs only infected with *P. multocida* (Halloy et al., 2005). Dietary exposure to fumonisin B1 in a concentration of 12,000 μ/kg feed increased the risk on PRRSv–associated disease (Ramos et al., 2010) and induced pulmonary edema which may aggravate *M. hyopneumoniae* infection (Pósa et al., 2013).

*Mycoplasma hyopneumoniae* is causing tremendous economic losses in all intensive pig producing countries worldwide (Maes et al., 2017), despite many attempts to control the disease (enzootic pneumonia) through vaccination strategies and control measures. Consequently, there is a high prevalence of both *M. hyopneumoniae* infections and a high contamination rate of feed with mycotoxins, more specific DON, in Europe (Rodrigues and Naehrer, 2012). Therefore, the odds for a pig to ingest feed contaminated with DON, whilst simultaneously being infected with *M. hyopneumoniae* is high. The present study aimed to investigate the effect of in-feed administration of DON, 3-acetylDON and 15-acetylDON at a moderately high level of 1,540 μg/kg feed, on the clinical course of an experimental *M. hyopneumoniae* infection with two genetically different *M. hyopneumoniae* strains in weaned piglets.

## Material and methods

## Study animals and Experimental design

The study was compliant with all relevant institutional and European standards for animal care and experimentation. The experiment was approved by the Ethics Committee for Animal Experiments of the Faculty of Veterinary Medicine and Faculty of Bioscience Engineering, Ghent University (approval number EC2015/112). Fifty *M. hyopneumoniae*-free Rattlerow-Seghers piglets (RA-SE Genetics NV, Ooigem, Belgium) were included in the study. The herd of origin has been free of *M. hyopneumoniae* and PRRSv since 2012 based on repeated serological testing, absence of clinical signs and pneumonia lesions, and nested polymerase chain reaction (nPCR) testing on tracheobronchial swabs as described in Chapter 3.4. The gilts and sows in the herd were vaccinated against *Erysipelothrix rhusiopathiae* and Parvovirus before insemination. No vaccinations were administered to the piglets. The piglets were weaned on average at 26 days of age and moved four days later to the experimental facilities of the Faculty of Veterinary Medicine, Ghent University, Belgium. The piglets were individually identified by means of an ear tag. The study design, the different parameters and timing are summarized in table 1. Upon arrival (D0) the piglets were randomly allocated to four different groups: 1) negative control group (NCG; n=5): sham-inoculated D8, D9 + control diet, 2) DON-contaminated group (DON; n=15): sham-inoculated D8, D9 and DON-diet (1,514 μg/kg), 3) DON-contaminated and *M. hyopneumoniae*-inoculated group (DONMHYO; n=15): experimentally inoculated with *M. hyopneumoniae* D8, D9 and DON-diet (1,514 μg/kg), 4) *M. hyopneumoniae*-inoculated group (MHYO; n=15): experimentally inoculated with *M. hyopneumoniae* D8, D9 + control diet. The number of 15 animals in the treatment groups enabled to find a difference of 5.26±4.7 in the main parameter namely macroscopic lung lesions (two-sided test) with 95%

certainty and a statistical power of 80%. This difference is biologically relevant and was based on previous research in our research group (Vicca et al., 2003). The NCG (5 pigs) was used to verify whether the purchased piglets remained *M. hyopneumoniae* negative throughout the study. The different groups were housed in four different facilities equipped with absolute filtered chambers (HEPA U15) in order to avoid cross-infection of *M. hyopneumoniae* between the different groups. The pigs had free access to drinking water and were fed *ad libitum*.

**Table 1: Experimental design, sample collections and timing in the different experimental groups**

| **Study day, D** | **Groups** | | | |
|---|---|---|---|---|
| | **NCG (n=5)** | **DON (n=15)** | **DONMHYO (n=15)** | **MHYO (n=15)** |
| D0[1] | Arrival | | | |
| | Weight | | | |
| | Randomisation | | | |
| D0-D35/36[2] | Commercial feed *ad libitum* | DON-contaminated feed *ad libitum* | DON-contaminated feed *ad libitum* | Commercial feed *ad libitum* |
| D1-D35[3] | RDS | | | |
| D8 | Sham-inoculation[4] | Sham-inoculation[4] | F7.2C[5]-inoculation | F7.2C[5]-inoculation |
| | Weight | | | |
| | Blood | | | |
| D9 | Sham-inoculation[4] | Sham-inoculation[4] | F1.12A[6]-inoculation | F1.12A[6]-inoculation |
| D21 | BALF | | | |
| | Blood | | | |
| D35/36[2] | Weight | | | |
| | Necropsy | | | |
| | Lung sample | | | |
| | BALF | | | |

[1]the average age of the pigs at arrival was 26 days,[2]all pigs of the NCG and DON group, and five pigs of MHYO were necropsied at D35. Ten pigs of MHYO and all pigs of DONMHYO were necropsied at D36, [3]RDS was not determined at D0 and D36 because the piglets arrived later than 8 a.m. (hour of performing coughing score every day) and part of the piglets were already euthanized on D36, respectively, [4]sham-inoculation was performed with sterile Friis medium, [5]highly virulent strain of *M. hyopneumoniae*, [6]low virulent strain of *M. hyopneumoniae*

NCG: negative control group, DON: deoxynivalenol contaminated group, DONMHYO: deoxynivalenol contaminated +*M. hyopneumoniae*-inoculated group, MHYO: *M. hyopneumoniae*-inoculated group, RDS: respiratory disease score, BALF: bronchoalveolar lavage fluid

## *Mycoplasma hyopneumoniae* strains and challenge infection

The pigs were inoculated with two different strains of *M. hyopneumoniae*: a highly virulent strain F7.2C and low virulent strain F1.12. Both strains had been differentiated and characterized at proteomic level with Sodium-Dodecyl-Sulphate Polyacrylamide gelelectrophoresis (SDS-page) (Calus et al., 2007) and at genomic level with Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), PCR-Random Fragment Length Polymorphism (PCR-RFLP) of the p146 gene, Variable Number of Tandem Repeats (VNTR) analysis of p97, with Multiple-Locus of VNTR Analysis (MLVA) (Stakenborg et al., 2005; Stakenborg et al., 2006; Vranckx et al., 2011a) and used in previous studies (Chapter 3.4; Vicca et al., 2003a; Villarreal et al., 2009). Previous research has shown that pigs are often infected with two or even three genetically different *M. hyopneumoniae* strains (Chapter 3.1; Vranckx et al., 2011; Vranckx et al., 2012), and in slaughter pigs infected with different strains, more lung lesions can be detected (Chapter 3.1). Therefore, the pigs in the present study were inoculated with two genetically different *M. hyopneumoniae* strains, to mimic the situation in the field. All pigs were anesthetised via the intramuscular route with 0.22 ml/kg BW of a mixture of tiletamine, zolazepam (Zoletil 100®, Virbac, Louvain-la-Neuve, Belgium) and xylazine (Xyl-M® 2%, VMD, Arendonk, Belgium) and the pigs of the DONMHYO and MHYO groups were endotracheally inoculated with 7 ml of inoculum containing $10^7$ colour changing units per ml (CCU/ml) of strain F7.2C on D8 and 7 ml of inoculum containing $10^7$ CCU/ml of strain F1.12A on D9. On both inoculation days, the pigs of the NCG and DON group were endotracheally sham-inoculated with 7 ml of sterile Friis medium. At D35 or D36 of the experiment, the pigs were euthanized using deep anaesthesia by intramuscularly administering 0.3 ml/kg BW of a mixture of tiletamine, zolazepam (Zoletil 100®, Virbac, Louvain-la-Neuve, Belgium) and xylazine (Xyl-M® 2%, VMD, Arendonk, Belgium), followed by exsanguination. For practical reasons and to avoid *M. hyopneumoniae* contamination of the samples, all pigs of the NCG and DON group were euthanized at D35, followed by five animals of the MHYO group. All the other animals of the MHYO and all animals of the DONMMHYO group were necropsied on D36.

## Deoxynivalenol contaminated diet

A commercial antibiotic-free diet for weaned piglets was purchased (Leievoeders N.V., Waregem, Belgium). Before purchasing the feed, a sample of the batch was tested with liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Monbaliu et al. (Monbaliu et al., 2010; Monbaliu et al., 2009) for the presence of DON, 3-Acetyldeoxynivalenol (3-ADON), zearalenone and fumonisin B1+ B2, the levels were below the reporting limit of 50 μg/kg, 50 μg/kg, 10 μg/kg and 50 μg/kg, respectively. The piglets of the NCG and MHYO groups were fed this commercial diet from D0 until D35/36. A part of the purchased feed (1,300 kg) was transported to the laboratory of the Department of Applied Biosciences (Faculty of Bioscience Engineering, Ghent University) to add the target concentration of 1,800 μg/kg feed DON. This procedure was followed: the reference strain *Fusarium graminearum (F. graminearum)* MUCL 42841 (Mycothèque de l'Université catholique de Louvain) was used to produce the DON-culture. The strain was grown in liquid mineral (MIN) medium supplemented with L-arginin as a selective nitrogen source, as previously described by Gardiner et al.

(Gardiner et al., 2009). After 14 days of cultivation, the culture was filtered and centrifuged. The obtained concentration of DON was determined with LC-MS/MS by adding 150 µl of the resulting undiluted MIN medium to 5 g of certified blank wheat standard (Sigma Aldrich, Overijse, Belgium). In total, 7,535 mg/kg DON was quantified and 1,076 mg/kg acetylated DON (3-ADON +15-ADON) in the grown DON-culture. Next to the presence of DON, the inoculum was tested with LC-MS/MS for the presence of other *F. graminearum* trichothecenes such as nivalenol, neosolaniol, fusarenon-X, diacetoxyscirpenol, HT-2 toxin and T-2 toxin, and results were below the detection limit. Also no zearalenone was detected. Eight liters (l) of inoculum were obtained for preparation of 1,300 kg of DON-contaminated feed in a concentration aimed at twice the recommended maximum pig feed level of 900 µg/kg or 1,800 µg/kg DON. First, 2.67 l of the inoculum was mixed with 10 kg of feed to obtain a thoroughly mixed premix of the DON-contaminated feed. Subsequently, the premix with inoculum was added to 433.3 kg of feed in a feed mill and thoroughly mixed for at least 40 minutes. The same procedure was repeated twice, to obtain the total amount of 1,300 kg of DON-contaminated feed in a concentration of 1,800 µg/kg and the feed was collected again in the original 25 kg bags of the feeding company. After preparation of the contaminated feed, a mixed sample originating from three DON-contaminated feed bags was taken and was submitted for LC-MS/MS to obtain the true DON-concentration of the contaminated feed. The results of the LC-MS/MS of the contaminated feed were 407 ± 120 µg/kg DON, 280 ± 100 µg/kg 3-ADON and 827 ± 300 µg/kg 15-ADON, resulting in a total DON and acetylated DON (3-ADON +15-ADON-concentration of 1,514 µg/kg in the contaminated feed sample. This feed was used in the DON and DONMHYO-groups from D0 until D35/36 of the study.

## Clinical and performance parameters

From D0 until D35/36 onwards, the piglets were observed daily at 8 a.m. for at least half an hour by the same researcher to assess appetite, faecal consistency and presence of dyspnea and tachypnea. A faecal consistency score was used to evaluate the faeces found on the pen floor before cleaning (Pedersen and Toft, 2011): 1 (firm and shaped), 2 (soft and shaped), both addressed as a normal faecal consistency in pigs, 3 (loose) and 4 (watery), with scores 3 and 4 considered as abnormal. A respiratory disease score (RDS) was recorded daily from D1 until D35. The score could range from 0-6 with 0 (no coughing), 1 (mild coughing after encouraged move), 2 (mild coughing in rest), 3 (moderate coughing after encouraged move), 4 (moderate coughing in rest), 5 (severe coughing after encouraged move), 6 (severe coughing in rest) (Halbur et al., 1996). The RDS was not determined at D0 and D36, as the pigs arrived later than 8 a.m. at the facilities and already part of the animals was euthanized on D35. The daily RDS values were averaged for the following periods: D1-7, D8-35/36 and D1-35/36. All pigs were weighed (kg) at the day of arrival (D0), the first inoculation day (D8) and the day of euthanasia (D35/36). The average daily gain (ADG, kg/pig/day) was calculated from D0-7, D8-35/36 and D0-35/36 by subtracting the starting weights from the final weights, divided by the number of days during that period.

## Macroscopic and histopathologic lung lesions

The lungs were removed and macroscopic lung lesions (MLL) (D35/36) were determined according to Hannan et al. (1982) (Hannan et al., 1982) from each pig. Consequently, the lungs were transported to the laboratory of the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University from the necropsy rooms from the experimental facilities in the same department and from each lung in each pig, samples from the right apical, cardiac and diaphragmatic lung lobes were collected. In case a lesion was present, a sample was collected including both healthy and affected lung tissue, at the border of the lesion. The samples were fixed in 10% neutral formalin and processed and embedded in paraffin for histopathological examination. Staining was performed with hematoxylin and eosin and the samples were scored using light microscopy based on the degree of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation (cuffing) (Morris et al., 1995). The scoring system ranged from 1 to 5 with 1) limited infiltration of macrophages and lymphocytes around bronchioles, with airways and alveolar spaces free of cellular exudates; score 2) light to moderate infiltrates with mild diffuse cellular exudates into airways; score 3, score 4 and score 5 (respectively mild, moderate and severe lesions characteristic of broncho-interstitial pneumonia, centered around bronchioles but extending to the interstitium, with lymphofollicular infiltration and mixed inflammatory cell exudates). Scores 1 and 2 are not considered to be related with *M. hyopneumoniae* infection, while scores 3 to 5 are presumptive of a *M. hyopneumoniae* infection (Del Pozo Sacristán, 2014; Morris et al., 1995). The percentage of air (percentage of lung area occupied by air) was examined by means of an automated image analysis system (Leica application suite AF Lite (Diegem, Belgium) and image J (Bethesda, Maryland, USA)) (Rasband, 1997-2016). This parameter is inversely proportional to the lymphohistiocytic infiltration in the lung tissue and the intrabronchiolar and bronchial exudate (Vicca et al., 2003b).

## Quantitative PCR for *Mycoplasma hyopneumoniae*

Two weeks post inoculation (PI) (D21) of the high virulent strain F7.2C (D8), bronchoalveolar lavage (BAL) fluid from all pigs, while conscious was collected. The pigs were snared and the mouth was opened by means of a gag to allow insertion of a catheter (Portex® Dog Catheter with Female Luer Mount, Smiths Medical International Ltd. Kent, United Kingdom). Next, the lungs were flushed with 10 milliliters (ml) of sterile phosphate buffered saline (PBS) and the fluid was subsequently aspirated. Additionally, at necropsy (D35/36), BAL fluid was collected from all pigs. Before collection of the histopathological samples, the head bronchus of the left part of the lung was flushed with 10 ml of sterile PBS. The BAL fluids were stored at -70°C awaiting analysis. The DNA was extracted with the DNeasy Blood & Tissue kit (QIAGEN, Qiagen Benelux, B.V., Antwerp, Belgium) with the DNA Purification protocol for bloods or bloody fluids (spin protocol) on 200 µl of BAL fluid according to the manual instructions and quantitative PCR (qPCR) was performed as previously described to detect the number of *M. hyopneumoniae* organisms (Marois et al., 2010). Briefly, after DNA-extraction, qPCR was performed with primers p102f (5'GTCAAAGTCAAAGTCAGCAAAC 3') and p102r (5'AGCTGTTCAAATGCTTGTCC 3') using SensiMixTM SYBR (Bioline GmbH, Luckenwalde, Germany) in

the CFX384 real-time PCR detection system (Bio-Rad, Nazareth, Belgium). A tenfold dilution series of *M. hyopneumoniae* DNA of strain F7.2C was used to convert the threshold values to the number of *M. hyopneumoniae* organisms. Values below the dilution of $1.50x10^1$ (1.18 log copies) were considered as negative (Marois et al., 2010; Vranckx et al., 2011b).

## Serology

The blood samples were stored at 4°C for a maximum of 24 hours. Subsequently the blood was centrifuged at 2000 rpm for 30 minutes and the serum was collected. The serum was stored at -70°C until analysis. At D8, D21 and at necropsy (D35/36), blood was collected from all pigs and tested with a blocking ELISA (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit, Oxoid Limited, Hampshire, UK) for presence of antibodies against *M. hyopneumoniae*, by following the instructions in the protocol manual. Sera with optical density < 50% of the average value of the OD-buffer control were considered to be positive. All values above or equal to 50 % of the average value of the OD-buffer control were classified as negative.

## Routine bacteriological culture on bronchoalveolar lavage fluid

For bacteriological examination, 10 microliters (µl) of BAL fluid collected at necropsy (D35/36) of each pig was inoculated on Columbia agar supplemented with 5% sheep blood (Oxoid, Hampshire, UK) with a *Staphylococcus pseudintermedius* streak (Villarreal et al., 2011). Plates were incubated for 48 h in a 5% $CO_2$-enriched environment at 35 °C for identification of respiratory bacteria in the lungs. All macroscopically different colonies were identified to the species level (score value >2.000) with a Bruker Daltonic Microflex LT Biotyper Biotyper MALDI-TOF mass spectrometer by using the direct transfer method and α-cyano-4-hydroxycinnamic acid (HCCA) as matrix, according to the manufacturer's guidelines. The spectra were obtained and analysed with the MBT Compass software version 3.1. (Bruker Daltonik), which included a database of 6,903 mean spectra projections.

## Statistical analyses

The independent variable in the statistical analyses was 'group': NCG, DON, DONMHYO and MHYO-group. The dependent variables were RDS, weight, ADG, MLL, histopathology and percentage of air, qPCR-results, percentage of *M. hyopneumoniae* qPCR-positive samples, *M. hyopneumoniae* specific AB expressed in OD values and percentage of ELISA *M. hyopneumoniae* positive samples. These variables were all run in separate models with 'pig' as statistical unit and no additional factors included into the model. The normality of the data was investigated by means of descriptive statistics, except for the binary data (*M. hyopneumoniae* qPCR positive samples and ELISA *M. hyopneumoniae* positive samples). The parameters BW, ADG and percentage air analysis were normally distributed and a one-way analysis of variance (ANOVA) test was used. In case of the RDS, a repeated measures ANOVA was performed. Scheffé's post-hoc test was used to make pair-wise comparisons. The qPCR-results and *M. hyopneumoniae* specific antibodies were not normally distributed and therefore, a non-parametric Kruskal-Wallis

test was used, with the Dunn-Bonferroni approach to make pair-wised comparisons, as well for MLL and histopathology results. In case of the normally distributed data, the mean and standard deviation (SD) were reported, in case of the non-parametric data, the median and the interquartile range were reported. All analyses for these parameters were performed with SPSS 23 for Windows (SPSS inc. Illinois, USA). Percentage of seropositive pigs and percentage of pigs testing positive with qPCR in each group were analyzed using binomial logistic regression (R version 3.3.1) (Team, 2016). The results were considered to be statistically significant when $P<0.05$.

## Results

## Clinical and performance parameters

In none of the groups, feed refusals or vomiting were observed. No tachypnea, nor dyspnea were observed in any of the groups. Post-weaning diarrhoea was observed from D0 onwards until D3 in all groups, therefore all pigs were treated IM with Colistin sulphate (Colivet 'S', Prodivet, Eynatten, Belgium) from D0 onwards for 5 days, according to the product leaflet. The post weaning diarrhoea lasted until D8, D3, D3 and D7 in NCG, DON, DONMHYO and MHYO respectively. In three groups some pigs with faecal consistency score 3 were noticed throughout the study: in NCG two pigs at D20 and D23 respectively, in DON one pig at D13 and in DONMHYO two pigs at D20 and D23, respectively. In MHYO, normal faecal consistency was observed throughout the study. Coughing was not observed in the NCG. All results of the different time periods for this parameter in the study are shown in table 2 ($RDS_{1-7}$, $RDS_{8-35}$ and $RDS_{1-35}$). The RDS from the first inoculation day onwards until euthanasia ($RDS_{8-35}$) were $0.00 \pm 0.00$, $0.0071 \pm 0.028$, $1.04 \pm 0.82$, $1.14 \pm 0.92$ for NCG, DON, DONMHYO and MHYO, respectively ($P<0.001$). No statistically significant differences were obtained between all groups for D1-7. For D8-35 and D1-35 a statistically significant difference was obtained between the experimentally infected (DONMHYO and MHYO) and non-infected pigs (NCG and DON) ($P<0.001$). However, no statistically significant differences were obtained for DONMHYO and MHYO groups in each time period of the study (table 2). The daily course of $RDS_{1-35}$ for each group is shown in figure 1.

There were no significant differences between the groups for the parameter BW and the ADG during the different time periods (table 2).

## Macroscopic and histopathologic lung lesions

The MLL of the NCG, DON, DONMHYO and MHYO groups were $0.00 \pm 0.00$, $0.00 \pm 0.00$, $2.77 \pm 3.22$ and $5.87 \pm 7.32$, respectively ($P<0.001$). The histopathological lung lesions were $1.70 \pm 0.20$, $2.00 \pm 0.30$, $2.40 \pm 0.80$ and $2.40 \pm 0.90$ for the NCG, DON, DONMHYO and MHYO group, respectively ($P<0.001$). The percentage of air was $45.94 \pm 6.54$, $46.85 \pm 6.86$, $49.75 \pm 9.90$ and $46.96 \pm 8.51$ for NCG, DON, DONMHYO and MHYO, respectively ($P=0.25$). There were no statistically significant differences between the DONMHYO and MHYO groups for these above-mentioned parameters , however there were statistically significant differences between

the experimentally infected (DONMHYO and MHYO) and non-infected pigs (NCG and DON) (P<0.001), except for the parameter percentage of air analysis (P=0.25) (table 2).

## Quantitative PCR for *Mycoplasma hyopneumoniae*

The samples of the NCG and DON group remained negative throughout the study. The qPCR results at D21 were 0.049 ± 0.37, 0.63 ± 0.81, 3.92 ± 2.60 and 3.24 ± 1.91 (P<0.001) and at D35/36 0.80 ± 0.70, 0.29 ± 1.09, 4.05 ± 1.38 and 4.20 ± 1.00 (P<0.001) for NCG, DON, DONMHYO and MHYO, respectively. There were no significant differences between the DONMHYO and MHYO group, however there were statistically significant differences between the experimentally infected (DONMHYO and MHYO) and non-infected pigs (NCG and DON) (P<0.001) for D21 and D35/36 (table 3).

## Serology

The serological results are presented in table 3. All pigs of the NCG and DON remained serologically negative throughout the study. The OD-values of the serological results at D35/36 were 1.15 ± 0.27, 1.16 ± 0.18, 0.25 ± 0.18 and 0.23 ± 0.094 for the NCG, DON, DONMHYO and MHYO, respectively. At necropsy (D35/36), all pigs of DONMHYO and MHYO were serologically positive.

## Routine bacteriological culture on bronchoalveolar lavage fluid

Few colonies of *Streptococcus suis* were isolated from one pig of both the DONMHYO (1/15) and MHYO (1/15) groups. In addition, few colonies of *Bordetella bronchiseptica* were isolated from one pig of DON (1/15) and four pigs of MHYO (4/15).

**Table 2. Results of the clinical parameters, macroscopical and microscopical lung lesions in the different experimental groups:**

Respiratory disease score (RDS), bodyweight, average daily weight gain (ADG), macroscopical lung lesions (MLL), histopathology score of the lungs and percentage of air in the lungs for NCG, DON group, DONMHYO group, MHYO group.

| Parameter | Groups | | | | |
|---|---|---|---|---|---|
| | **NCG (n=5)** | **DON (n=15)** | **DONMHYO (n=15)** | **MHYO (n=15)** | **P-value** |
| **RDS** | | | | | |
| D1-7 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.00 |
| D8-35 | 0.00 ± 0.00[a] | 0.0071 ± 0.028[a] | 1.04 ± 0.82[b] | 1.14 ± 0.92[b] | <0.001 |
| D1-35 | 0.00 ± 0.00[a] | 0.0057 ± 0.025[a] | 0.83 ± 0.84[b] | 0.91 ± 0.94[b] | <0.001 |
| **Weight ± SD (kg)** | | | | | |
| D0 | 6.37 ± 1.09 | 6.39 ± 0.92 | 6.38 ± 1.04 | 6.42 ± 1.23 | 1.00 |
| D8 | 7.70 ± 1.13 | 8.04 ± 1.27 | 8.20 ± 1.08 | 8.11 ± 1.90 | 0.92 |
| D35/36 | 20.10 ± 2.33 | 19.45 ± 3.83 | 21.02 ± 3.18 | 21.75 ± 4.36 | 0.38 |
| **ADG (kg/pig/day)** | | | | | |
| D0-8 | 0.52 ± 0.14 | 0.53 ± 0.190 | 0.56 ± 0.15 | 0.67 ± 0.17 | 0.12 |
| D0-35/36 | 0.39 ± 0.057 | 0.37 ± 0.11 | 0.41 ± 0.075 | 0.43 ± 0.098 | 0.41 |
| D8-35/36 | 0.46 ± 0.076 | 0.42 ± 0.13 | 0.46 ± 0.094 | 0.49 ± 0.10 | 0.37 |
| **MLL, histopathology and percentage of air D35/36** | | | | | |
| MLL | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 2.77 ± 3.22[b] | 5.87 ± 7.32[b] | <0.001 |
| Histopathology | 1.70 ± 0.20[a] | 2.00 ± 0.30[a] | 2.40 ± 0.80[b] | 2.40 ± 0.90[b] | <0.001 |
| Percentage of air (%) | 45.94 ± 6.54 | 46.85 ±6.86 | 49.75 ±9.90 | 46.96 ± 8.51 | 0.25 |

The parameters bodyweight, ADG, percentage of air analysis were analysed by means of one way analysis of variance, the parameter RDS with a repeated measures analysis of variance, in both cases with Scheffé post-hoc test to make pair-wised comparisons, therefore means ± SD are reported. The parameter MLL and histopathology were analysed with Kruskal-Wallis test and the Dunn-Bonferroni approach to make pair-wised comparison, therefore medians and interquartile range are reported. Different superscripts in one row are statistically different (P<0.05).

NCG: negative control group, DON: deoxynivalenol contaminated group, DONMHYO: deoxynivalenol contaminated +*M. hyopneumoniae*-inoculated group, MHYO: *M. hyopneumoniae*-inoculated group, SD: standard deviation

n: number

D=Day of the study

ADG: average daily gain

RDS: respiratory disease score

MLL: macroscopic lung lesions

**Fig. 1: Course of average respiratory disease score (RDS) from day 1 until the day of necropsy (D 35)**

Average RDS from D1 until D35 in the negative control group (NCG), the DON-contaminated group (DON), DON-contaminated and *M. hyopneumoniae*-inoculated group (DONMHYO), and *M. hyopneumoniae*-inoculated group (MHYO). The challenge infections were performed on D8 (a highly virulent *M. hyopneumoniae* strain F7.2C) and D9 (a low virulent strain F1.12) in DONMHYO and MHYO. The NCG and DON were sham-inoculated with sterile Friis medium on D8 and D9.

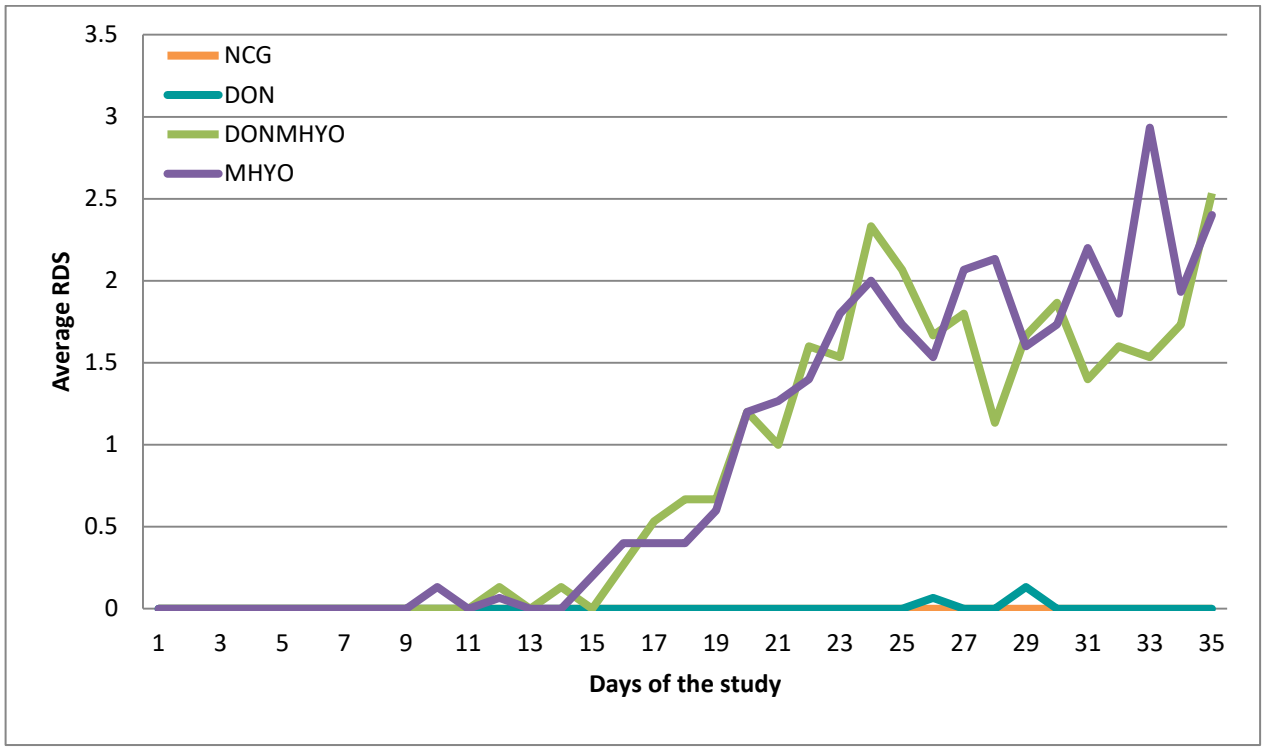

**Table 3: Results of *Mycoplasma hyopneumoniae*-DNA detection in the BALF and serology in the different experimental groups:**

qPCR-results (log copies of *M. hyopneumoniae* DNA/ml BALF), percentage of *M. hyopneumoniae* qPCR positive samples, *M. hyopneumoniae* specific antibodies expressed in OD-values, percentage of ELISA *M. hyopneumoniae* positive samples and routine bacteriological examination in BALF in the different groups NCG, DON group , DONMHYO group and MHYO group.

| **Parameter** | **Groups** | | | | |
|---|---|---|---|---|---|
| | **NCG (n=5)** | **DON (n=15)** | **DONMHYO (n=15)** | **MHYO (n=15)** | **P-value** |
| **qPCR (log copies of *M. hyopneumoniae* DNA /ml BALF) ± SD** | | | | | |
| D21 | 0.049 ± 0.37$^{a}$ | 0.63 ± 0.81$^{a}$ | 3.92 ± 2.60$^{b}$ | 3.24 ± 1.91$^{b}$ | <0.001 |
| D35/36 | 0.80 ± 0.70$^{a}$ | 0.29 ± 1.09$^{a}$ | 4.05 ± 1.38$^{b}$ | 4.20 ± 1.00$^{b}$ | <0.001 |
| **Percentage of *M. hyopneumoniae* qPCR positive samples (n of positive samples/total n of samples tested)** | | | | | |
| D21 (%) | 0.00 (0/5)$^{a}$ | 0.00 (0/15)$^{a}$ | 86.67 (13/15)$^{b}$ | 93.33 (14/15)$^{b}$ | <0.001 |
| D35/36 (%) | 0.00 (0/5)$^{a}$ | 0.00 (0/15)$^{a}$ | 100.00(15/15)$^{b}$ | 100.00 (15/15)$^{b}$ | <0.001 |
| ***M. hyopneumoniae* specific AB expressed in OD-values ± SD** | | | | | |
| D8 | 1.68 ± 0.23 | 1.83 ± 0.34 | 1.71 ± 0.19 | 1.70 ± 0.19 | 0.27 |
| D21 | 1.15 ± 0.22$^{a}$ | 1.29 ± 0.23$^{a}$ | 1.16 ± 0.36$^{ab}$ | 0.94 ± 0.26$^{b}$ | 0.001 |
| D35/36 | 1.15 ± 0.27$^{a}$ | 1.16 ± 0.18$^{a}$ | 0.25 ± 0.18$^{b}$ | 0.23 ± 0.094$^{b}$ | <0.001 |
| **Percentage of ELISA *M. hyopneumoniae* positive samples** (n of positive samples/total n of samples tested) | | | | | |
| D8 (%) | 0.00 (0/5) | 0.00 (0/15) | 0.00 (0/15) | 0.00 (0/15) | 1.00 |
| D21 (%) | 0.00 (0/5) | 0.00 (0/15) | 0.00 (0/15) | 13.33 (2/15) | 0.17 |
| D35/36 (%) | 0.00 (0/5)$^{a}$ | 0.00 (0/15)$^{a}$ | 100.00 (15/15)$^{b}$ | 100.00 (15/15)$^{b}$ | <0.001 |

The parameter qPCR and *M. hyopneumoniae* specific AB were analysed with a non-parametric Kruskal-Wallis and the Dunn-Bonferroni approach to make pair-wised comparisons, therefore median and interquartile range are reported. The prevalence of *M. hyopneumoniae* qPCR positive samples and the prevalence of ELISA *M. hyopneumoniae* positive samples were analysed with binomial logistic regression. Different superscripts in one row are statistically different (P<0.05).

NCG: negative control group, DON: deoxynivalenol contaminated group, DONMHYO: deoxynivalenol contaminated + *M. hyopneumoniae*-inoculated group, MHYO: *M. hyopneumoniae*-inoculated group

Different superscripts in one row are statistically different (P<0.05)

n: number

SD: standard deviation

D=Day of the study

*M. hyopneumoniae*: *Mycoplasma hyopneumoniae*

AB: antibodies

OD: optical densities

## Discussion

In the present study, the outcome of the main parameters (RDS, MLL, histopathological lesions and log copies of *M. hyopneumoniae* DNA in bronchoalveolar lavage fluid) was not statistically different in *M. hyopneumoniae* infected pigs that received or did not receive DON-contaminated feed. This indicates that ingestion of DON- and acetylated DON-contaminated feed at a concentration of 1,514 μg/kg, exceeding the maximum guidance level for pigs according to EU regulation, did not aggravate the severity of an experimental *M. hyopneumoniae*-infection in weaned piglets.

The challenge infection was successful as all animals in the *M. hyopneumoniae* inoculated groups (MHYO and DONMHYO group) coughed, showed lung lesions (except for one animal in the MHYO group), seroconverted and *M. hyopneumoniae*-DNA was detected in BAL fluid at necropsy. The results obtained in the *M. hyopneumoniae* challenged pigs (DONMHYO and MHYO group) differed significantly from the non-challenged pigs (DON group, NCG). The latter pigs remained serologically negative throughout the entire study period and no *M. hyopneumoniae*- DNA was detected in BAL fluid two weeks post-inoculation, nor at necropsy. The obtained data in the challenged groups were comparable with a study performed by our research group with the same challenge model (Chapter 3.4), demonstrating the repeatability of the model. The two *M. hyopneumoniae*-strains were not administered on the same day, as the infection model practiced at our research group uses seven ml inoculum containing $10^7$ CCU of the strain/ml (Vicca et al., 2003). It is not known whether piglets of four to five weeks of age (on average 26 days, D0 at arrival and inoculation at D8) are able to cope with twice the inoculum volume (14 ml for both strains). In addition, it is not known whether both strains will grow next to each other, or whether one strain will overgrow the other strain consuming most of the nutrients, when these *M. hyopneumoniae* strains are grown in the same culture flask. Consequently, it was decided to challenge the piglets on two consecutive days.

In the present study, DON was aimed to be administered at two times the maximum guidance level of DON advised by the European commission for pigs (1,800 μg/kg feed) (Communities, 2006). This dose was selected as Madson et al. (Madson et al., 2014) stated that moderate (1,000-5,000 μg/kg feed) to high concentrations (> 5,000 μg/kg feed) are associated with delayed or supressed immune responses due to leukocyte apoptosis and resulting in increased disease susceptibility (Madson et al., 2014; Pestka, 2008). It was demonstrated that a dose-related decrease in daily feed intake is observed when administering DON-supplemented feed to pigs, and that this effect was mostly significant to the control feed in the range of 2,000 μg/kg – 4,000 μg/kg (Bergsjø et al., 1992; Dänicke et al., 2004; Prelusky et al., 1994). Therefore, a dose below this range was chosen to be administered to the pigs in this study to limit feed refusals and to avoid hampering of DON possibly influencing the investigated parameters. The commercial, un-spiked feed was tested before the start of the study, not only for the presence of DON, but also for zearalenone and fumonisin B1+B2, to avoid these components having an effect in the feed of the control groups (MHYO group, NCG) and avoiding an additional or synergistic effect to the DON-toxicity in the DON-spiked groups (DON group, DONMHYO group) (Pestka, 2008). After preparation of the contaminated feed, a mixed feed sample from three DON-contaminated feed bags was submitted to LC-MS/MS to detect the DON-concentration and to ensure thorough mixing of the DON-

inoculum in the feed. The results of the LC-MS/MS was 1,514 μg/kg of DON and acetylated forms and deviated only slightly from the target concentration of 1,800 μg/kg. Cytotoxicity of the produced DON in the study was not tested on beforehand, as the deleterious effects of DON in the pigs are known and the same DON inoculation method in pig feed was already successfully used in a study by Goossens et al., 2012. In the latter study, in the *in vivo* effect of DON on intestinal damage influencing the resorption of doxycycline was determined in pigs.

It remains to be elucidated why no impact of DON and acetylated forms in the pigs was observed in the present study. One of those factors might be the degree of susceptibility of certain breeds/lines to the effects of DON (Eriksen and Pettersson, 2004). For instance a lower severity in porcine circovirus type 2 associated histopathological lesions has been shown for Piétrain compared to landrace pigs (Opriessnig et al., 2009), but so far no studies have assessed the impact of genetic differences in sensitivity to the effects of DON.

No feed-refusals were observed in the pigs administered the DON-contaminated feed. The minimum emetic dose for orally distributed DON in pigs is 100 μg/kg (Pestka et al., 2007), yet vomiting was not observed in this study. The reason why this did not occur is unclear. It must be noted that the observation period of the pigs (30 min. of RDS-scoring, 15 min. feeding and cleaning in the morning, 10 min. of observation and cleaning in the evening) was fairly short compared to the time the pigs spent in the facilities. However, the main researcher was always present after providing the feed to identify possible sick pigs, as most healthy pigs start eating immediately after providing the feed and Young et al., (Young et al., 1983) observed that vomiting occurs within minutes after ingesting the DON-contaminated feed. Pestka et al. (Pestka, 2007) stated that vomiting due to DON-contaminated feed, is more likely if the feed is ingested at once and not via smaller portions throughout the day. The contaminated feed and hence the DON and DON-acetylated forms in the DONMHYO and DON pigs, was consumed throughout the day as the pigs were fed *ad libitum* and had freely access to the feed.

The *M. hyopneumoniae*-infected pigs on the DON and DON-acetylated forms-supplemented feed did not have a higher RDS compared to the pigs only infected with *M. hyopneumoniae*. No other studies are available investigating the impact of DON on respiratory tract disease signs in *M. hyopneumoniae*-infected pigs. However the effect of fumonisin B1 in combination with *M. hyopneumoniae* or the effect of DON on other pathogens has been studied. Pósa et al., (2013) studied the effect of 20 000 μg/kg fumonisin B1 on *M. hyopneumoniae*-infected pigs, however no firm conclusions could be drawn regarding the difference in coughing between the *M. hyopneumoniae*-infected pigs with or without fumonisin B1 supplementation in the feed. Halloy et al. (2005) investigated the impact of fumonisin B1 administered orally (500 μg/kg BW per day, seven days) in *P. multocida*-infected pigs and concluded that these pigs coughed more compared to the pigs only infected with *P. multocida*.

It is known that *M. hyopneumoniae*-infections can negatively influence production parameters, such as ADG (Thacker and Minion, 2012). Deoxynivalenol-ingestion in pigs may result in reduced feed intake, and subsequently decrease ADG (Eriksen and Pettersson, 2004; Friend et al., 1982; Trenholm et al., 1983). In the present study, DON and DON-acetylated forms did not decrease ADG in the *M. hyopneumoniae* inoculated animals. This result is in agreement with Accensi et al. (2006) (840 μg DON/kg feed), Gerez et al. (2015) (1,500 μg DON/kg feed), and Savard et al. (2015b).The latter authors studied the impact of DON (2500 and 3,000 μg DON/kg feed) in pigs that were simultaneously infected with PCV2. Pósa et al., (Pósa et al., 2013), did not find

an effect of fumonisin B1 (20, 000 μg/kg) on ADG in *M. hyopneumoniae*-infected pigs. Rotter et al. (Rotter et al., 1994) observed an adaptation of pigs to oral ingestion of *Fusarium* mycotoxins from one week onwards. In that study, pigs were fed a diet mixed with naturally contaminated corn (28,700 μg/kg DON, 8,600 μg/kg 15-ADON and1,100 μg/kg ZEA ) to obtain DON-concentrations of 750, 1,500 or 3,000 μg DON /kg feed during 28 days. During the first week, the exposed pigs had lower weight gains than the pigs fed a non-contaminated control feed. At the end of the study, however, the overall weight gains did not differ anymore between these groups. This adaptation might be one of the reasons why we did no see an effect of DON and acetylated forms on daily growth. The relative low number of pigs, followed up during a limited period of time, which is inherent in experimental infection studies like ours, also makes it difficult to obtain a statistical significant difference in daily growth (Arsenakis et al., 2016; Jensen et al., 2002; Vicca et al., 2003). No effect of DON and acetylated forms ingestion on the macroscopic and histopathological lung lesions was observed in the *M. hyopneumoniae*-infected group. This finding is in agreement with Pósa et al (2011) who neither saw a statistical difference in macroscopic lung lesions between the *Bordetella bronchiseptica* and *Pasteurella multocida* dually-infected groups with or without supplementation of fumonisin B1 in the feed (10,000 μg/kg) of three-day-old piglets. Savard et al. (2015) on the other hand, did observe an effect of DON-supplementation in the feed (3,500 μg/kg, three weeks) on macroscopic lung lesions in PRRSv-infected pigs (Eriksen et al., 2004). In this study, DON and acetylated forms-ingestion did not influence the number of log copies detected in the *M. hyopneumoniae*-infected animals. Similarly, Savard et al. (Savard et al., 2015a) did not observe an effect of DON-contamination on the presence of viral RNA, measured with qPCR, in PRRSv-infected pigs. It is not clear why the obtained effects of dietary DON vary among experiments, it might be explained by different factors such as starting weight or age of the pigs, the contamination source of DON (natural versus artificial contamination), presence of other known or unknown undetected fungal metabolites or pathogens, duration of the study (adaptation), number of pigs used in the study, gender of the pigs, health status, nutritional balance of the pig and statistical design of the study (Dersjant-Li et al., 2003; Goyarts et al., 2005; Rotter et al., 1995). It is not known why DON and acetylated forms did not influence a *M. hyopneumoniae*-infection under the circumstances in this study. Deoxynivalenol has a good distribution in the lung of the pig (Prelusky and Trenholm, 1991). However, as *M. hyopneumoniae* is attached to the cilia of the upper respiratory tract and does not invade the parenchyma of the lung (Blanchard et al., 1992; Jaques et al., 1992; Maes et al., 2017), it might be that DON is not able to exert its effect on the pathogen. More research is needed to investigate this relationship in pigs, for instance by *in vitro* tests on pig tracheal explants. On the other hand discrepancies have been reported between *in vivo* (higher viremia in pigs exposed to DON in the feed ) and *in vitro* (decreased PRRSv replication in MARC-cells) effects of DON on PRRSv (Savard et al., 2015a; Savard et al., 2014), thus extrapolation of *in vitro* results to *in vivo* effects has to be done cautiously.

## Conclusions

No effect was observed of DON contamination in a moderately high dose in the feed on the severity of an experimental *M. hyopneumoniae*-infection in weaned piglets. In the field, however, the impact of DON-contaminated feed on a *M. hyopneumoniae*-infection might be more expressed, because mostly multi-mycotoxin contamination of the feed occurs (Antonissen et al. 2014; Opriessnig et al., 2009; Young et al., 1983; Pestka et al.,

2007), the pigs can be exposed to DON-contaminated feed for a longer period than the five week exposure period in this study, often suboptimal housing and climate conditions may prevail and other pathogens may be present (Thacker et al., 2012; Friend, 1982). Further research should assess the impact of DON on *M. hyopneumoniae* infections under these multi-pathogen and multi-mycotoxins circumstances and investigating the impact of DON *in vitro* on *M. hyopneumoniae* in tracheal explant cells. More research could also focus on factors influencing the effect on DON such as health status, gender, age, and possible genetic resistance of the pigs.

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# *3.4 Efficacy of one dose vaccination against experimental infection with two Mycoplasma hyopneumoniae strains*

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## Abstract

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary agent of enzootic pneumonia in pigs. Pigs are often infected with different *M. hyopneumoniae* strains. This study assessed the efficacy of vaccination against experimental infection with two genetically different *M. hyopneumoniae* strains in weaned piglets. At 33 days of age (D0), 45 *M. hyopneumoniae*-free piglets were randomly assigned to three different groups: 1) negative control group (NCG; n=5): not vaccinated, not infected, 2) positive control group (PCG; n=20): not vaccinated, infected, and 3) vaccination group (VG; n=20): single vaccination with an inactivated whole-cell *M. hyopneumoniae* vaccine (Hyogen®, Ceva) (D1), infected. The PCG and VG were endotracheally inoculated with 7 x $10^7$ CCU in 7 ml of the highly virulent *M. hyopneumoniae* strain F7.2C (D24) and 7 x $10^7$ CCU in 7 ml low virulent strain F1.12A (D25). A respiratory disease score (RDS) was assessed from D24 until D53. At D53 (euthanasia), macroscopic lung lesions (MLL) were scored, log copies of *M. hyopneumoniae* DNA (qPCR) and IL-1β and IL-6-concentrations (ELISA) on bronchoalveolar lavage fluid were determined

The RDS and MLL at euthanasia were respectively 0, 1.20 and 0.55 ($P<0.001$) and 0, 7.56 and 0.68 ($P<0.001$) for NCG, PCG and VG, respectively. The qPCR results for PCG and VG were 3.99 and 1.78 log copies ($P<0.001$), respectively, with a significant difference between PCG and VG. The IL-1β and IL-6 results at euthanasia for NCG, PCG and VG were 17.61, 1283.39 and 53.04 pg/ml ($P<0.001$) and 148.10, 493.35 and 259.80 pg/ml ($P=0.004$), respectively with a significant difference between PCG and VG.

Vaccination with Hyogen® in pigs was efficacious against an experimental challenge with both a low and highly virulent *M. hyopneumoniae* strain as the vaccinated pigs coughed significantly less, and showed significantly less lung lesions compared to the non-vaccinated challenged pigs: the vaccinated animals showed a 52.9 % lower RDS and 91.0 % lower MLL compared to the PCG. In the bronchoalveolar lavage fluid collected at the necropsy of the vaccinated pigs, a significantly lower amount of *M. hyopneumoniae*-DNA and a significantly lower IL-1β and IL-6 concentration was found compared to the pigs of the PCG.

## Introduction

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the causative agent of enzootic pneumonia (Maes et al., 2008). The disease has a worldwide impact on intensive swine production and causes substantial losses due to reduced growth of the pigs, poor feed conversion ratio, higher antimicrobial use and increased susceptibility to secondary respiratory agents (Maes et al., 1999; Maes et al., 2008; Villarreal et al., 2011b). Previous research has shown that most pigs are infected with more than one *M. hyopneumoniae* strain (Vranckx et al., 2011; Vranckx et al., 2012a), and in chapter 3.1 it was showed that a higher severity and prevalence of *Mycoplasma*-like lung lesions was found in batches of slaughter pigs with detection of more than one *M. hyopneumoniae* strain. It is generally accepted that control of *M. hyopneumoniae* is critical for reducing economic losses in infected swine operations (Pointon et al., 1985). This is achieved by optimizing housing conditions, management practices, antimicrobial therapy and vaccination against *M. hyopneumoniae* (Haesebrouck et al., 2004). It is estimated that 70 percent of swine herds are practising vaccination against *M. hyopneumoniae* worldwide (Martelli et al., 2014). Vaccination reduces clinical signs, lung lesions and improves performance, although colonization is not prevented and there is no significant reduction in transmission (Haesebrouck et al., 2004; Jensen et al., 2002; Kyriakis et al., 2001; Meyns et al., 2006; Villarreal et al., 2011b). The benefits obtained through vaccination can vary from herd to herd (Martelli et al., 2006). Most commercially available bacterin vaccines are based on an adjuvanted whole-cell preparation of an inactivated *M. hyopneumoniae* strain (Haesebrouck et al., 2004). This strain is mostly the J-strain of *M. hyopneumoniae*, which was isolated in 1958 (Goodwin and Whittlestone, 1963; Marchioro et al., 2013b). Recently a commercial bacterin became available, using *M. hyopneumoniae* strain 2940, isolated in the 1999's from a farm in the United States facing a severe outbreak of enzootic pneumonia. Also, most vaccination studies so far used one single strain for experimental infection. As most pigs are infected with more than one *M. hyopneumoniae* strain (Chapter 3.1; Vranckx et al., 2011; Vranckx et al., 2012a), it may be more appropriate to challenge the pigs with different *M. hyopneumoniae* strains.

The aim of the present study was to determine the efficacy of a commercial vaccine (Hyogen®) against an experimental challenge with two genetically different (low and highly virulent) *M. hyopneumoniae* strains.

## Material and methods

### Study animals and Experimental design

The study was performed after approval by the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University (approval number EC2014/165). Forty-five *M. hyopneumoniae*-free Rattrelow-Seghers (RA-SE Genetics NV, Ooigem, Belgium) piglets were enrolled in the study. The herd of origin has been free of *M. hyopneumoniae* for many years based on repeated serological testing, absence of clinical signs and pneumonia lesions, and nPCR testing on tracheobronchial swabs. The piglets were free of following pathogens as well: porcine reproductive and respiratory syndrome virus, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*. The piglets were weaned at 28 days of age and moved to the experimental facilities of the Faculty of Veterinary Medicine, Ghent University, Belgium. They were housed in experimental chambers with absolute filters (HEPA U15) and were fed *ad libitum* using a non- antimicrobial supplemented diet. At 33 days of age (D0), the piglets were randomly allocated to three different groups (D0): 1) negative control group (NCG; n=5): not vaccinated, not infected, 2) positive control group (PCG; n=20): not vaccinated, infected, and 3) vaccination group (VG; n=20): single vaccination with a one- dose commercial vaccine (Hyogen®, D1, CEVA Santé Animale, Libourne Cedex, France), infected.

Hyogen® is a whole-cell bacterin based on strain BA 2940-99, oil adjuvanted with paraffin and *Escherichia coli* J5 LPS with thiomersal as excipient. The animals of the VG were intramuscularly vaccinated with two ml of the commercial vaccine. The animals of the PCG and NCG were injected intramuscularly with two ml of phosphate-buffered saline (PBS) on D1.

### *Mycoplasma hyopneumoniae* strains and challenge infection

The highly virulent F7.2C and the low virulent *M. hyopneumoniae* strain F1.12A were used for challenge infection (Vicca et al., 2003). The pigs were anesthesized with 0.22 ml/kg of a mixture of Zoletil 100® (Virbac, Louvain la Neuve, Belgium) and Xyl-M® 2% (VMD, Arendonk, Belgium). The pigs of the PCG and VG were endotracheally inoculated with 7 $x10^7$ CCU in 7 ml inoculum of strain F7.2C at D24 and with 7 $x10^7$ CCU in 7 ml inoculum of strain F1.12A at D25. Pigs of the NCG were endotracheally inoculated with 7 ml sterile culture medium (Friis medium) at D24 and D25. At four weeks post-inoculation (PI) (D53), all piglets were euthanized. Therefore, deep anaesthesia was applied by intramuscularly administrating 0.3 ml/kg of a mixture of Zoletil 100® (Virbac, Louvain la Neuve, Belgium) and Xyl-M® 2% (VMD, Arendonk, Belgium), followed by exsanguination.

### Clinical and performance parameters

The piglets were observed for at least half an hour daily from D0 until D53. Body condition, appetite, manure consistence, and presence of dyspnea or tachypnea were evaluated. A respiratory disease score (RDS) was recorded daily by the same person at eight a.m. from D1 until D53 and could range from 0-6 with 0 (no coughing), 1 (mild coughing after encouraged move), 2 (mild coughing in rest), 3 (moderate coughing after

encouraged move), 4 (moderate coughing in rest), 5 (severe coughing after encouraged move), 6 (severe coughing in rest) (Halbur et al., 1996). The daily RDS values were averaged for the following periods: D1- D23, D24– D53, D1-D53. All pigs were weighed (g) at D0, the day of the first inoculation (D24) and the day of euthanasia (D53), to calculate the anesthesia dose discussed above. The average daily gain (ADG) (g/pig/d) from day 0-24, 24-53 and from day 0-53 was calculated according to Del Pozo Sacristán et al. (2014)(Del Pozo Sacristán et al., 2014) by subtracting the starting weights from the final weights divided by the number of days during the respective periods.

## Macroscopic and histopathologic lung lesions

The lungs were collected and scored for macroscopic lung lesions (MLL) (0-35) according to Hannan et al. (1982) (Hannan et al., 1982). Samples from the right apical, cardiac and diaphragmatic lung lobe were collected. If lesions were present, the samples were collected from the border of a lesion. Samples were fixated in 10% neutral formalin and processed and embedded in paraffin for histopathological examination. The samples were stained with hematoxylin and eosin and scored using light microscopy according to the degree of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation (cuffing) (Morris et al., 1995). The scoring system ranged from 1-5 with 1) limited infiltration of macrophages and lymphocytes around bronchioles, with airways and alveolar spaces free of cellular exudates; score 2) light to moderate infiltrates with mild diffuse cellular exudates into airways; score 3, score 4 and score 5 [respectively mild (score 3), moderate (score 4) and severe (score 5)] lesions characteristic of broncho-interstitial pneumonia, centered around bronchioles but extending to the interstitium, with lymphofollicular infiltration and mixed inflammatory cell exudates. Scores 1 and 2 are considered not to be related with *M. hyopneumoniae* infection, while scores 3 to 5 are presumptive of a *M. hyopneumoniae* infection. The percentage of lung area occupied by air (percentage air) was examined by means of an automated image analysis system (Leica application suite AF Lite (Diegem, Belgium) and image J (Bethesda, Maryland, USA)) (Rasband, 1997-2016). This parameter is inversely proportional to the lymphohistiocytic infiltration in the lung tissue and the intrabronchiolar-and bronchial exudate.

## Quantitative PCR for *Mycoplasma hyopneumoniae*

Bronchoalveolar lavage fluid was collected from all animals two weeks PI (D39) by inserting a catheter (Portex® Dog Catheter with Female Luer Mount, Smiths Medical International Ltd. Kent, United Kingdom) in the trachea by means of a mouth gag and whilst snaring the pigs. Next, the lungs were flushed with 20 mL sterile PBS to collect the bronchoalveolar lavage fluid. Additionally, during necropsy (D53), bronchoalveolar fluid was collected from the left part of the lung before collection of the histopathological samples by flushing the head bronchus of the left part of the lung with 20 mL sterile PBS. The bronchoalveolar lavage fluid was divided into seven aliquots and stored at -70°C awaiting analysis. From one aliquot of the bronchoalveolar lavage samples, DNA was extracted with the QIAGEN protocol (QIAGEN, DNeasy Blood & Tissue kit, Belgium) and quantitative PCR (qPCR) was performed as previously described to detect the numbers of *M. hyopneumoniae* organisms (Marois et al., 2010). A tenfold dilution series of *M. hyopneumoniae* DNA of strain F7.2C was used to convert the threshold

values to the number of *M. hyopneumoniae* organisms. Values below the dilution of $1.50x10^1$ (1.18 log copies) were considered as negative.

## Immunological parameters on bronchoalveolar lavage fluid

*M. hyopneumoniae- specific antibodies in bronchoalveolar lavage fluid*

The isotype of *M. hyopneumoniae*-specific antibodies in bronchoalveolar lavage fluid collected at D39 and D53 was determined via an indirect ELISA (expressed in optical density "OD"-values) according to the protocol of Bereiter et al. (Bereiter et al., 1990; Marchioro et al., 2013a). Briefly, a Nunc Maxisorp® flat-bottom 96 well plate (eBioscience, Vienna, Austria) was coated with Tween® 20 extracted *M. hyopneumoniae* antigens on which the bronchoalveolar lavage fluid (D39, D53) was added, undiluted. Peroxidase labeled goat anti-porcine IgA or IgG polyclonal antibodies (Bethyl Laboratories, Texas, TX, USA) were added and the OD at 450 nm was measured.

*Cytokines in bronchoalveolar lavage fluid*

The bronchoalveolar lavage fluid collected at D39 and D53 was tested undiluted for presence of porcine TNF-α (TNF-α Swine Antibody Pair, Invitrogen), IL-1β (Porcine IL-1 beta/IL-1F2 DuoSet, R&D Systems) and IL-6 (Porcine IL-6 DuoSet, R&D Systems). A sandwich ELISA was performed according to the manufacturers' recommendations. The sample reactions were measured using OD at 450 nm and quantified by the use of a standard curve as described in the manual.

## Serology

At D1, D24 and at euthanasia (D53), blood was collected from all pigs and tested for the presence of antibodies against *M. hyopneumoniae* with a blocking ELISA (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit, Oxoid Limited, Hampshire, UK), according to the protocol manual. The blood samples were stored at 4°C for a maximum of 24 hours. Subsequently the blood was centrifuged at 2000 rpm for 30 minutes and the serum was collected. The serum was stored at -70°C until analysis. Sera with optical density < 50% of the average value of the OD-buffercontrol were considered to be positive. All values above or equal to 50 % of the average value of the OD-buffercontrol were classified as negative.

## Routine bacteriological culture and bronchoalveolar lavage fluid

For the bacteriological examination, ten µL of bronchoalveolar lavage fluid collected on D53 of each pig was inoculated on Columbia agar supplemented with 5% sheep blood (Oxoid, Hampshire, UK) with a *Staphylococcus pseudintermedius* streak (Villarreal et al., 2011a). Plates were incubated overnight in a 5% $CO_2$-enriched environment at 35 °C for 48 h for identification of respiratory bacteria in the lungs.

## Statistical analyses

Descriptive statistics were performed in order to check the normality of the data. One-way analysis of variance (ANOVA) was used to analyse weight and ADG. Repeated measurements ANOVA was performed to analyze the RDS data. Scheffé's post hoc test was used to make pairwise group comparisons. The parameters MLL, histopathological lesions, percentage air analysis, ELISA *M. hyopneumoniae*, TNF-α, IL-1β, IL-6, Ig A, Ig G and qPCR results were analyzed using a non-parametric Kruskal-Wallis, as the data did not fulfil the assumptions of normality. Theses analyses were performed with SPSS 22 for Windows (SPSS inc. Illinois, USA). Percentage of seropositive pigs and percentage of pigs testing positive with qPCR in each group were analyzed using binomial logistic regression (R version 3.3.1) (Team, 2016). Results were considered to be statistically significant when $P<0.05$.

## Results

No animals died during or shortly after the inoculations. One out of 45 piglets in the NCG was euthanized (D22) (Release®300 mg/ml, WDT, Garbsen, Germany) due to nervous symptoms and lateral decubitus. Necropsy was performed and *H. parasuis* was isolated in pure culture from the meninges and pericardium. All animals in the PCG coughed, showed macroscopic lung lesions and seroconverted. *Mycoplasma hyopneumoniae*-DNA was detected in 19 out of 20 animals from this group.

## Clinical and performance parameters

The results of the clinical parameters are summarized in table 1. There was no coughing in the NCG throughout the study. The $RDS_{24-53}$ was 1.20 ± 0.83 and 0.55 ± 0.42 for the PCG and VG ($P<0.001$), respectively. This corresponds with a reduction of 52.9% in RDS when vaccinating the piglets compared to the PCG. All groups significantly differed from each other. These analyses and the $RDS_{1-53}$ and $RDS_{1-23}$ results are shown in table 1 and figure 1.

The average group weight at D0, D24 and D53 and the ADG calculated during these three periods (0-24, 24-53 and 0-53 days of age) for each group are shown in table 1.

## Macroscopic and histopathologic lung lesions

There were no macroscopic lung lesions in the NCG. The MLL of the PCG and VG was 7.56 ± 4.73 and 0.68 ± 0.73, respectively ($P<0.001$) (Table 1). A reduction of 91.0% in MLL of the VG was observed compared to the PCG. The histopathological lung lesion scoring in the NCG, PCG and VG was 1.31 ± 0.18, 3.32 ± 0.85 and 1.92 ± 0.47 ($P<0.001$), respectively. The percentage of lung area occupied by air (percentage air) was 47.72 ± 7.29, 34.49 ± 8.22 and 45.23 ± 5.99 % for the NCG, PCG and VG ($P<0.001$), respectively. For the MLL and percentage of air analysis, the PCG was significantly different from the VG and NCG. For the histopathology score, all groups significantly differed from each other (Table 1).

## Quantitative PCR for detection of *Mycoplasma hyopneumoniae* DNA

The samples of the NCG remained negative throughout the study. The number of log copies of *M. hyopneumoniae* detected in the bronchoalveolar lavage fluid with qPCR in the PCG and VG were: 3.99 ± 1.20 (PCG) and 1.78±1.36 (VG) (P<0.001) at D53 (Table 1). The results of D39 and the percentage of animals that tested positive by qPCR are also shown in table 1.

**Fig. 1: Average respiratory disease score (RDS) during the trial for the negative control group (NCG), positive control group (PCG) and vaccination group (VG)**

The average RDS was assessed daily from day 1 until day 53. The piglets were vaccinated at D1 and the inoculations were performed at D24 and D25.

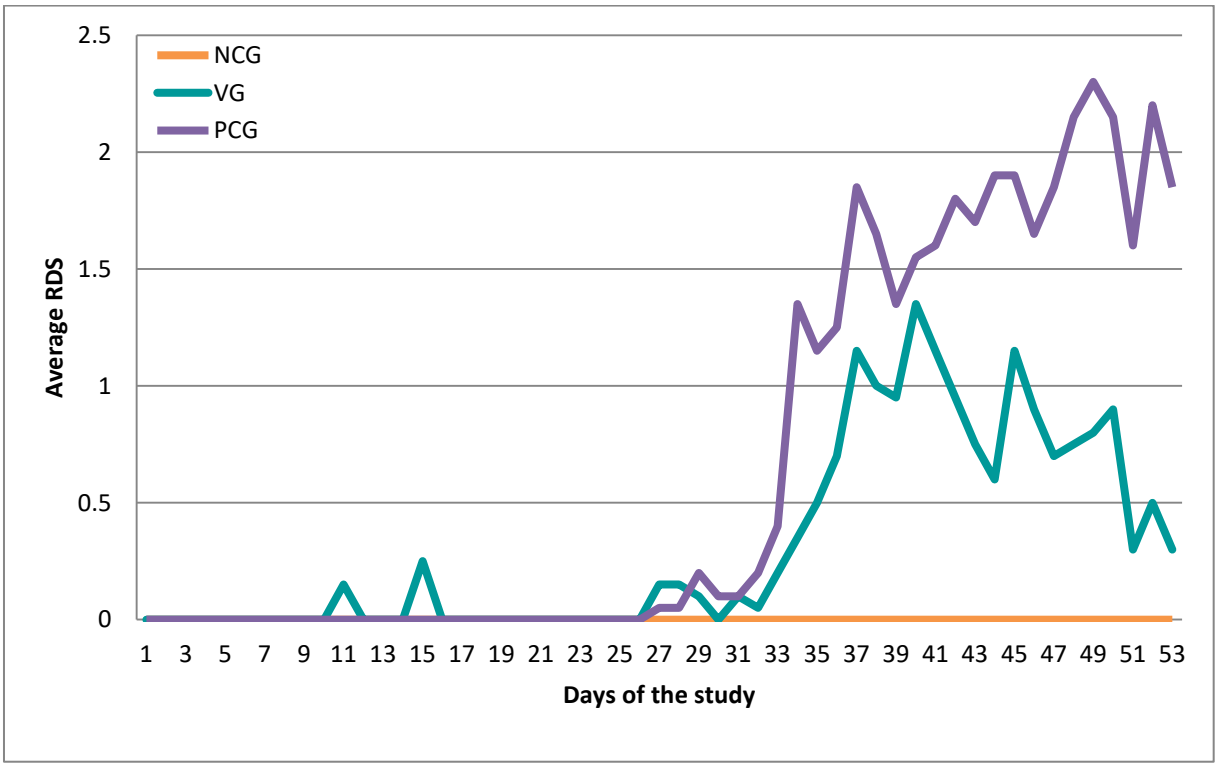

**Table 1: Results of different parameters in the different experimental groups:** the respiratory disease score (RDS), body weight, average daily gain (ADG), *M. hyopneumoniae* specific antibodies, macroscopical lung lesions (MLL), histopathology and air analysis, log copies of *M. hyopneumoniae* DNA (qPCR), percentage of of *M. hyopneumoniae* qPCR positive samples, *M. hyopneumoniae* specific AB expressed in OD-values and Percentage of ELISA *M. hyopneumoniae* positive samples

| Parameter | Experimental Groups | | | |
|---|---|---|---|---|
| | **NCG (#=5)** | **PCG (#=20)** | **VG (#=20)** | **P-value** |
| **RDS** | | | | |
| D1-53 | 0 ± 0 [a] | 0.68 ± 0.41 [b] | 0.32 ± 0.86 [c] | *<0.001* |
| D1-23 | 0 ± 0 [a] | 0 ± 0 [a] | 0.017 ± 0.060 [a] | 0.983 |
| D24-53 | 0 ± 0 [a] | 1.20 ± 0.83 [b] | 0.55 ± 0.42 [c] | *<0.001* |
| **Weight ± SD** | | | | |
| D0 | 7.04 ± 1.22[a] | 6.97 ± 0.84 [a] | 7.04 ± 1.02 [a] | 0.966 |
| D24 | 15.25 ± 3.96 [a] | 16.39 ± 2.16 [a] | 15.61 ± 2.96 [a] | 0.581 |
| D53 | 36.51 ± 8.63 [a] | 34.63 ± 6.63 [a] | 34.27 ± 5.35 [a] | 0.808 |
| **ADG (g/pig/d)** | | | | |
| D0-24 | 357 ± 123 [a] | 393 ± 62.8 [a] | 357 ± 92.0 [a] | 0.372 |
| D24-53 | 733 ± 194 [a] | 629 ± 199 [a] | 643 ± 102 [a] | 0.504 |
| D0-53 | 563 ± 144 [a] | 522 ± 121 [a] | 514 ± 87.0 [a] | 0.714 |
| **MLL, histopathology and air analysis D53** | | | | |
| MLL | 0 ± 0 [a] | 7.56 ± 4.73 [b] | 0.68 ± 0.73 [a] | *<0.001* |
| Histopathology | 1.31 ± 0.18 [a] | 3.32 ± 0.85 [b] | 1.92 ± 0.47 [c] | *<0.001* |
| Percentage of air (%) | 47.72 ± 7.29 [a] | 34.49 ± 8.22 [b] | 45.23 ± 5.99 [a] | *<0.001* |
| **log copies of *M. hyopneumoniae* DNA/ml BALF (qPCR) ± SD** | | | | |
| D39 | 0.36 ± 0.64 [a] | 1.81 ± 1.32 [a] | 1.23 ± 1.61 [a] | 0.062 |
| D53 | 0.40 ± 0.43 [a] | 3.99 ± 1.20 [b] | 1.78 ± 1.36 [a] | *<0.001* |
| **Percentage of *M. hyopneumoniae* qPCR positive samples in BALF (#positive samples/total number of samples)** | | | | |
| D39 | (0/4) 0[a] | (14/20) 70[b] | (7/20) 35[c] | *<0.01* |
| D53 | (0/4) 0[a] | (19/20) 95[b] | (12/20) 60[c] | *<0.001* |
| ***M. hyopneumoniae* specific AB expressed in OD-values ± SD in serum** | | | | |
| D1 | 1.51 ± 0.037 [a] | 1.48 ± 0.058 [a] | 1.48 ± 0.16 [a] | 0.578 |
| D24 | 0.91 ± 0.044 [a] | 0.95 ± 0.069 [a] | 0.50 ± 0.17 [b] | *<0.001* |
| D53 | 0.90 ± 0.10 [a] | 0.23 ± 0.11 [a] | 0.087 ± 0.041 [b] | *<0.001* |
| **Percentage of ELISA *M. hyopneumoniae* positive samples in serum** | | | | |
| D1 | (0/5) 0[a] | (0/20) 0[a] | (0/20) 0[a] | 1.000 |
| D24 | (0/4) 0[a] | (0/20) 0[a] | (15/20) 75[b] | *<0.001* |
| D53 | (0/4) 0[a] | (20/20) 100[b] | (20/20)100[b] | *<0.001* |

Different superscripts a, b, c in one row are statistically different (P<0.05)
NCG: negative control group, PCG: positive control group, VG: vaccination group, SD: standard deviation, D=Day of the study, ADG: average daily gain, kg: kilogram, RDS: respiratory disease score, *M. hyopneumoniae*: *Mycoplasma hyopneumoniae*
AB: antibodies, OD: optical densities, #: number, MLL: macroscopic lung lesions, qPCR: quantitative polymerase chain reaction, DNA: DeoxyriboNucleic Acid, BALF: bronchoalveolar lavage fluid

## Immunological parameters on bronchoalveolar lavage fluid

All results of the immunological parameters on D39 and D53 are summarized in table 2 .

**Table 2: Immunological parameters measured in the BALF at D39 and D53**

| Parameter | Experimental groups | | | |
|---|---|---|---|---|
| | **NCG (#=5)** | **PCG (#=20)** | **VG (#=20)** | **P-value** |
| **Ig G ± SD (OD-values)** | | | | |
| D39 | 0±0 [a] | 0.069±0.14 [a] | 0.31±0.65 [a] | 0.366 |
| D53 | 0.38±0.17 [a] | 2.25±0.91 [b] | 2.41±0.88 [b] | *0.005* |
| **Ig A ± SD (OD-values)** | | | | |
| D39 | 0±0 [a] | 0.067±0.18 [a] | 0.55±0.99 [a] | 0.359 |
| D53 | 0.18±0.14 [a] | 2.10±0.75 [b] | 2.08±0.58 [b] | *0.005* |
| **TNF-α ± SD (pg/ml)** | | | | |
| D39 | 23.61±1.74 [a] | 18.56±4.40 [a] | 21.12±4.56 [a] | *0.029* |
| D53 | 16.03±1.10 [a] | 14.53±5.95 [a] | 15.15±2.40 [a] | 0.065 |
| **IL-1β ± SD (pg/ml)** | | | | |
| D39 | 32.47±1.77 [a] | 239.63±511.35 [a] | 247.13±606.71 [a] | 0.367 |
| D53 | 17.61±1.98 [a] | 1283.39±1075.65 [b] | 53.04±54.23 [a] | *<0.001* |
| **IL-6 ± SD (pg/ml)** | | | | |
| D39 | 130.33±18.65 [ab] | 242.24±122.23 [a] | 164.64±84.48 [b] | 0.03 |
| D53 | 148.10±8.89 [a] | 493.35±494.70 [b] | 259.80±333.38 [a] | *0.004* |

D39: two weeks after challenge
D53: necropsy
Different superscripts a, b, ab in one row are statistically different (P<0.05)
NCG: negative control group, PCG: positive control group, VG: vaccination group
SD: standard deviation
D=Day of the study
OD: optical densities
#: number

*Detection of M. hyopneumoniae- specific antibodies in bronchoalveolar lavage fluid*

There were no significant differences in the Ig G and Ig A results between the three groups at D39 (Table 2). At D53, the Ig G values were 0.38 ± 0.17, 2.25 ± 0.91 and 2.41 ± 0.88 for the NCG, PCG and VG, respectively (P=0.005). At D53, the results for Ig A were 0.18 ± 0.14, 2.10 ± 0.75, 2.08 ± 0.58 for the NCG, PCG and VG, respectively (P=0.005). Both the Ig G and Ig A concentrations at D53 of the PCG and VG differed significantly from the NCG.

*Detection of cytokines in bronchoalveolar lavage fluid*

The TNF-α, IL-1β and IL-6 results at D39 and D53 are shown in table 2. There were no significant differences obtained in the TNF-α- results (pg/ml) at D39, nor at D53.

At D53, the IL-1β concentration in the bronchoalveolar lavage fluid was 17.61 ± 1.98, 1283.39 ± 1075.65 and 53.04 ± 54.23 pg/ml in the NCG, PCG and VG, respectively (P<0.001).

At D39, the IL-6 concentration was significantly different between the PCG and VG (Table 2). At D53, the IL-6 concentration was 148.10 ± 8.89, 493.35 ± 494.70 and 259.80 ± 333.38 for the NCG, PCG and VG, respectively (P=0.004) with significantly higher concentrations in the PCG compared to the NCG and VG.

## Serology

The serological results are presented in Table 1. All pigs of the NCG remained serologically negative throughout the study (D1-D53) and at necropsy (D53) all pigs of the PCG and VG were serologically positive for *M. hyopneumoniae*. The OD values at D53 were 0.90 ± 0.10, 0.23 ± 0.11 and 0.087 ± 0.041 in the NCG, PCG and VG, respectively (P<0.001). In the VG significantly lower OD-values were detected, thus higher *M. hyopneumoniae* specific antibodies compared to the PCG and NCG. (Table 1).

## Bacteriological culture

*Streptococcus suis* was isolated in only one pig of the PCG. No relevant bacterial growth was observed in all other lung samples.

## Discussion

This study showed that the vaccine was efficacious for the most part against an experimental challenge with both a low and highly virulent *M. hyopneumoniae* strain. The vaccinated pigs coughed significantly less, showed significantly less lung lesions, a significantly lower number of log copies in the bronchoalveolar fluid at euthanasia was shown and the IL-1β and IL-6 concentrations at euthanasia were lower compared to the non-vaccinated inoculated pigs.

The challenge infection was performed with two genetically different (Stakenborg et al., 2005) *M. hyopneumoniae* isolates. The virulence of these strains was evaluated by Vicca et al. (2003) and both strains were characterised at proteomic and genomic level by Calus et al. (2007), Vranckx et al. (2011) and Stakenborg et al. (Stakenborg et al., 2006), respectively. The double challenge infection model was successful as all animals in the PCG showed lung lesions, coughed and seroconverted. In 19 out of 20 animals of the PCG at euthanasia, DNA of *M. hyopneumoniae* was detected in the bronchoalveolar lavage fluid. It is well known that there can be quite some variation between pigs in terms of responses to an experimental infection. Why this particular pig tested negative is not known. Possibly, the conditions for multiplication of the *M. hyopneumoniae* strains were not optimal in that pig. Although bronchoalveolar fluid is an appropriate sampling technique to detect *M. hyopneumoniae* during the early stages of infection, it fails to recover some positive animals in case of chronic infection (Bandrick et al., 2014). Taken these factors into consideration, this might be the reason why, although the pig was challenged, it did not test positive. Although comparing to other experimental settings should be done with caution, clinical symptoms and lung lesions were comparable or more severe than the single F7.2C challenge model performed in our research group (Arsenakis et al., 2016; Del Pozo Sacristán et al., 2012; Marchioro et al., 2014; Marchioro et al., 2013a; Meyns et al., 2006; Meyns et al., 2007; Meyns et al., 2004; Vicca et al., 2005; Vicca et al., 2003; Villarreal et al., 2009; Villarreal et al., 2011; Vranckx et al., 2012b) and the standard deviation was lower (Arsenakis et al., 2016; Del Pozo Sacristán et al., 2012; Marchioro et al., 2014; Vicca et al., 2003; Villarreal et al., 2009; Vranckx et al., 2012b). It was shown that most pigs in the field are simultaneously infected with two or sometimes three genetically different *M. hyopneumoniae* strains and that when batches of slaughter pigs are infected with more than one *M. hyopneumoniae* strain, this can result in more (severe) pneumonia lesions and fissures (Chapter 3.1). The current experimental setting might therefore better resemble the field situation than infection with a single strain. Finally, it was shown that anesthetizing and inoculating the piglets with seven ml of inoculum on two consecutive days is feasible, as none of the piglets showed adverse reactions or died during or shortly after the inoculations.

The pigs in the vaccinated group coughed significantly less (-53%) from inoculation until euthanasia compared to the positive control group, demonstrating the efficacy of the vaccine. The degree of coughing is an important efficacy parameter (Baskerville, 1972; Sarradell et al., 2003) not only under experimental but also under field conditions. A coughing index can be used to estimate if a higher prevalence of enzootic pneumonia in a herd might be present (Nathues et al., 2012) and to determine the most optimal timing of serological sampling of the pigs or collecting bronchoalveolar fluid to be tested with PCR (Leon et al., 2001; Nathues et al., 2012). In this study, no statistical significant differences were observed in the parameters weight and ADG, although the pigs

in the VG grew slightly faster compared to the PCG from day of inoculation until day of euthanasia onwards. Although the parameter weight and ADG are of great use to be evaluated under field circumstances, in experimental trials they are merely of descriptive value as the low number of animals used, the duration of the trial and the sometimes high standard deviations are the reason why only numerical differences are found (Arsenakis et al., 2016; Jensen et al., 2002; Vicca et al., 2003).

The MLL score in the vaccinated pigs was very low (0.68) and statistically different from the PCG (7.56), implying a reduction with 91%, and demonstrating the efficacy of the vaccine. A recent field study showed that when pigs are infected with more strains, more lung lesions are detected (Chapter 3.1). When comparing the MLL scores from the double inoculated challenge in PCG with those from challenged unvaccinated pigs in the single inoculation model from previous studies (highest MLL was 6.69 for F7.2C challenge in Villarreal et al., (Villarreal et al., 2011a), higher MLL in this study are obtained. However, comparing different experimental settings, should be done with caution. To make a true comparison, the resulting MLL from the double infection model should be compared with the single inoculation model from both strains F7.2C and F1.12A in one experimental setting.

In the vaccinated animals significantly less lymphohistiocytic infiltration (1.92) was observed compared to the PCG (3.32). These results are in accordance with the results of previous studies (Arsenakis et al., 2016; Marchioro et al., 2014; Villarreal et al., 2011a). Although many aspects of the effect of vaccination on the immunological response remain unclear (Thacker et al., 2000b), the present results confirm that vaccination has a regulating function on the immune system, causing less infiltration of macrophages in the lung tissue (Vranckx et al., 2012). A significantly lower area occupied by air or air percentage was measured in the non-vaccinated animals of PCG (34.49%) compared to VG (45.23%). Infection with *M. hyopneumoniae* results in intrabronchiolar- and bronchial exudate and infiltration of lymphohistiocytic cells in the lung tissue. These responses may result in compressing the airways and alveoli, leading to less air volume in the lungs (Marchioro et al., 2013b). Vaccination also significantly reduced the log copies of *M. hyopneumoniae* DNA compared to the non-vaccinated animals (1.78 versus 3.99), which may lead to less shedding of *M. hyopneumoniae* in vaccinated pigs. Similar reductions were found in previous studies (Vranckx et al., 2012b) (Woolley et al., 2014). These results, although very promising, confirm once more that vaccination is not able to prevent pigs from being colonized as commonly known (Haesebrouck et al., 2004).

*Mycoplasma hyopneumoniae*-specific antibody (IgG and IgA) levels in tracheo-bronchial washings were not significantly different between vaccinated and non-vaccinated pigs. Our findings are in accordance with Djordevic et al. (1997) (Djordjevic et al., 1997; Marchioro et al., 2013a), but Thacker et al. (2000) suggested that secretion of *M. hyopneumoniae*- antibodies induced by vaccination into the bronchoalveolar washings might be implied in resolving mycoplasmal pneumonia (Thacker et al., 2000a; Thacker et al., 2000b; Thacker et al., 2000c). Further research is needed to clarify a possible protective role of *M. hyopneumoniae*-specific antibodies in trachea-bronchial washings.

There were no significant differences between the vaccinated and non-vaccinated animals for the TNF-α concentration in the bronchoalveolar fluids. In the study of Marchioro et al. (Marchioro et al., 2013a),

vaccination significantly decreased TNF-α concentrations, but the difference was small and only borderline significant. This highlights the need to further elucidate the pathogenesis of *M. hyopneumoniae* and the exact mechanisms in which way *M. hyopneumoniae*-bacterins provide protection to the animal.

The cytokines IL-1β and IL-6, next to TNF-α are pro-inflammatory cytokines (Yang et al., 2004). Previous research stated that they mediate lymphocyte infiltration and activation in the pneumonic lung (Ahn et al., 2009; Van Reeth et al., 2002), and thus are associated with the induction of pneumonia lesions (Asai et al., 1993; Marchioro et al., 2013a; Rodríguez et al., 2004). The pro-inflammatory cytokines TNF-α, IL-1β and IL-6 were correlated with MLL at D39 and D53. TNF-α was weakly and not significantly correlated with MLL on both sampling days (D39 and D53). Interleukin-1ß was strongly and significantly correlated with MLL at euthanasia, and IL-6 was moderately and significantly correlated with MLL on D39 and D53 (data not shown). Vaccinating the *M. hyopneumoniae* infected pigs, resulted in a lower infection level in these vaccinated pigs, a lower production of pro-inflammatory cytokines IL-1ß and IL-6 (but not TNF-α), and subsequently less damage in the lung. The IL-1β concentration and the standard deviation in the non- vaccinated pigs was very high. This result is in accordance with the result of Meyns et al. (2007) at 28 days post inoculation with the F7.2C isolate. One can conclude that a part of the piglets in the non- vaccinated group showed very high IL-1β levels. The reason why this occurred is not clear, however the individual susceptibility of the pig to an *M. hyopneumoniae*-infection must be kept into account causing the challenge infection to be more successful. The IL-1β and IL-6 concentrations at necropsy in the vaccinated pigs were significantly lower than in the non-vaccinated pigs and not significantly different from the levels in negative control group. These results substantiate the statement that was made in Marchioro et al. (2013a) based on the IL-1β result of the vaccinated pigs at D36 that IL-1β and IL-6 may have a regulatory role upon *M. hyopneumoniae* vaccination.

In this study, the pigs were vaccinated approximately 4 weeks before challenge, as vaccination is most likely to be effective if active immunity is established before the piglets are exposed to the pathogen. Under field conditions, however, the time between vaccination and infection is not known, and very likely highly variable between pigs. Hence, it is possible that the vaccine used in the field is less efficacious when the timing of vaccination is not optimal. As this is an experimental study, the results cannot be extrapolated as such to field conditions. Under field circumstances, more factors challenging the efficacy of a vaccine are present, compared to the controlled environment of an experimental facility. First, multi-factorial and multi-pathogen enzootic pneumonia outbreaks are typically found in the field, rather than isolated *M. hyopneumoniae* outbreaks (Maes et al., 1999). The piglets originated from a herd free of important diseases which can influence the outcome of an *M. hyopneumonia*e infection, such as PRRSv *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae* (Maes et al., 2008; Sibila et al., 2009; Steenhard et al., 2009) and according to some authors infection with certain pathogens can determine the effectiveness of *M. hyopneumoniae* vaccination as well (Martinod, 1996; Steenhard et al., 2009; Stevenson, 1998). Second, the impact of weaning stress should be taken into account when vaccinating piglets at weaning age in the field. Although the impact of weaning stress on vaccine efficacy might not be clear (Arsenakis et al., 2016), it could not have influenced the efficacy of the vaccine, as the pigs were transported and vaccinated some days after weaning. Thirdly, passively acquired maternal derived immunity could not have influenced the efficacy of the vaccine as these piglets were originating from a herd free of *M. hyopneumoniae* and no vaccinations against *M.*

*hyopneumoniae* in the sows, nor the piglets were performed. This is a double sided given. On the one hand, Hodgins et al. (Hodgins et al., 2004) stated that maternal antibodies in the piglets were associated with reduced antibody responses to vaccination. On the other hand, the piglets in the study could not benefit from the passively transferred cell-mediated immunity. Bandrick et al. (Bandrick et al., 2014) showed that vaccination of piglets against *M. hyopneumoniae* in the face of antigen-specific maternal-derived immunity results in cell-mediated immunity priming and anamnestic cell-mediated immunity responses following the exposure *to M. hyopneumoniae* antigen. On the other hand, some factors in the experimental study were comparable to field circumstances or challenged the vaccine more. First, although the piglets were free from certain pathogens, other than that, the piglets originated from a conventional farm. Second: the piglets were transported from the herd of origin to the experimental facilities and the piglets needed to establish a new hierarchy after comingling. These events are associated with stress, which might influence the efficacy of the vaccine. This can resemble the stress that piglets experience in the field, when sorted and moved from one facility to another in a multi-site production system. Thirdly: the vaccine was highly challenged in this experimental study, because as stated by Villarreal et al. (2009) and Meyns et al. (2004), it can be assumed that the *M. hyopneumoniae* challenge dose used to infect the pigs was higher than might be reached under natural conditions resulting in a faster and higher colonization level and presumably challenging the vaccine more than under field circumstances. Although extrapolation of the efficacy testing of the vaccine results obtained in experimentally *M. hyopneumoniae* infected pigs should be done with caution, this infection model enabled to study the effect of the vaccine in *M. hyopneumoniae* infected pigs in a reproducible and standardized way.

## Conclusions

Using a double challenge infection model with a low and highly virulent *M. hyopneumoniae* strain, one-dose vaccination of piglets was clinically efficacious, as coughing was reduced by more than 50% and macroscopical lung lesions by 91%. In addition, the lymphohistiocytic infiltration in lung tissue was lower, the number of log copies of *M. hyopneumoniae* DNA detected in bronchoalveolar lavage fluid and the IL-1β and IL-6 concentrations at euthanasia were lower in the vaccinated animals compared to non-vaccinated animals.

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# CHAPTER 4: GENERAL DISCUSSION

# *General Discussion*

# GENERAL DISCUSSION

*Mycoplasma hyopneumoniae* is the primary agent involved in enzootic pneumonia and plays a key role in the porcine respiratory disease complex as well. The pathogen is considered to be a leading cause of economic losses to swine producers worldwide (Thacker and Minion, 2012). Many factors involved in the clinical course of the disease and the economic losses, are known. These factors are management practices, interactions with other both viral and bacterial respiratory agents (Opriessnig et al., 2011), antimicrobial treatment and vaccination (Maes et al., 2008). The impact of other factors such as *M. hyopneumoniae* strain diversity, indoor pollutants components and, mycotoxins in the feed was less clear at the start of this PhD. The objective of this thesis was to investigate several of these less studied factors. In addition, as the search to control the disease continues, new vaccines against *M. hyopneumoniae* are being developed. Therefore, one chapter focussed on the efficacy of a new bacterin on a challenge infection with *M. hyopneumoniae*.

In this present chapter, the results and implications of the different studies will be discussed, general conclusions will be presented and perspectives for further research will be provided.

## 4.1. *Mycoplasma hyopneumoniae* diversity

### 4.1.1. Portraying the diversity of *Mycoplasma hyopneumoniae* in slaughter pigs from Flemish pig farms

Numerous reports regarding *M. hyopneumoniae* genetic diversity have been described, however no agreement has been obtained between different studies regarding the presence of a limited number or a higher number of *M. hyopneumoniae* strains at herd level (Burgher Pulgarón et al., 2015; Charlebois et al., 2014; Dos Santos et al., 2015; Marois-Créhan et al., 2012; Mayor et al., 2007; Nathues et al., 2011; Pantoja et al., 2016; Takeuti et al., 2017; Tamiozzo et al., 2015; Vranckx et al., 2011; Vranckx et al., 2012b). On top of that, most research focusses on displaying a snapshot of the present *M. hyopneumoniae* strains within a herd at a certain time point. Only one report investigates repeated samplings from the same farms. In that study, from a limited number of farms three slaughter batches within the same farm were investigated (Vranckx et al., 2011). It was found that the same strains persisted in two out of three herds. However, in one herd this was not the case and two different strains were detected at the next two slaughter points. It was interesting to explore this more in depth in a higher number of farms. Therefore, *M. hyopneumoniae* diversity from three slaughter batches originating from ten farms was studied in chapter 3.1.

In chapter 3.1, we demonstrated that *M. hyopneumoniae* strains are highly diverse. Out of the 495 nPCR positive BALF samples, 135 different *M. hyopneumoniae* strains were obtained in the entire dataset. The average number of different strains per batch was seven with a minimum of one and a maximum of 13. At herd level on average 14 different *M. hyopneumoniae* strains with a minimum of 6 and a maximum of 23 were detected. In each batch of each herd new strain introductions were observed compared to the previous batches: on average 4.6 (ranging from 2-8) new strains were detected when batch 2 was compared with batch 1 and on average 2.7 (ranging from

1-5) new strain introductions were detected when comparing batch 3 with the previous two batches in each herd. The possible reason why a much higher diversity of *M. hyopneumoniae* was observed in our study compared to the above mentioned studies will be explained in the following section.

Extrapolation of *M. hyopneumoniae* diversity results from non-endemically *M. hyopneumoniae* to endemically infected pig populations such as in Belgium should be done with caution. Lower *M. hyopneumoniae* circulation will most probably result in a lower *M. hyopneumoniae* diversity. It might be expected that when infection pressure is low, *M. hyopneumoniae* strains are pressurised less on using genetic diversity as a tool to escape the immunity of the host. Mayor et al. (2007) concluded that a single strain was responsible for an outbreak of enzootic pneumonia when investigating multiple samples from different farms. All field samples however originated from Switzerland, a region where *M. hyopneumoniae* was eradicated and only sporadic reinfections occur (Hege et al., 2002; Nathues et al., 2011). In the study of Nathues et al. (2011) it was concluded that herds and pigs were colonised with different *M. hyopneumoniae* strains. These results were obtained in North Western Germany, a region endemically infected with *M. hyopneumoniae* with a similar pig density compared to Belgium (grosse Beilage et al., 2009) and were more comparable with the results obtained in chapter 3.1 of the current thesis.

In general the more regions of the genome are investigated, the higher the chance to detect differences between *M. hyopneumoniae* strains. Indeed, in the studies of Mayor et al. (2007), Pantoja et al. (2016) and Takeuti et al. (2017), respectively one, two and two VNTR's were examined, while in our study four VNTR's were examined. On the other hand, in the study of Vranckx et al. (2011; 2012b) four VNTR regions were examined, and a lower diversity was observed compared to our results. Therefore other factors such as herd characteristics might be involved in finding a higher diversity in *M. hyopneumoniae* strains.

In Takeuti et al. (2017), where a lower *M. hyopneumoniae* diversity was observed in the herds compared to our findings, two of the three herds did not purchase any gilts, the third farm did purchase gilts, but they had a *M. hyopneumoniae* negative status. In Vranckx et al. (2011) a higher frequency of purchasing gilts was indeed suggested to be involved in the higher diversity seen in one of the three herds. In the study in chapter 3.1, more than half of the farms purchased breeding gilts on a regular basis, which may have influenced the number of *M. hyopneumoniae* strains entering the farms. The stocking density of piglets in the nursery may be important as well, as a higher stocking density might result in a higher transmission of *M. hyopneumoniae* (Stärk, 2000). In Vranckx et al. (2012) the stocking density in the nursery was 0.3 $m^2$/animal. In the study in chapter 3.1, half of the farms had a stocking density lower than 0.3 $m^2$/animal, which may have influenced the higher diversity detected in this study.

Vranckx et al al. (2011) suggested that all-in/all-out-management practices might be involved as well in the occurrence of a higher *M. hyopneumoniae* strain diversity. In the study of Vranckx et al., (2012b) all herds were practicing an all-in/all-out -management in the nursery and finishing units. In the study of chapter 3.1 all farms except one practised all-in/all-out however in the finishing units, four out of ten farms did not. This might as well be one of the plausible factors why a higher *M. hyopneumoniae* diversity was encountered in our study.

Vaccination of the piglets against *M. hyopneumoniae* might be of importance as well. The decision not to vaccinate against *M. hyopneumoniae* can be made because there is a low infection level and there are no *M. hyopneumoniae*-related problems in the farm (Maes et al., 2008; Maes et al., 2003). A good external biosecurity in the herd might

prevent entrance of new *M. hyopneumoniae* strains resulting in less circulation. As at least 70% of the herds are implementing vaccination against *M. hyopneumoniae* (Maes et al., 2008), our study aimed to investigate diversity in farms vaccinating their piglets against *M. hyopneumoniae*, while in the study of Vranckx et al. (2011; 2012b) farms were selected based on not vaccinating the piglets against *M. hyopneumoniae*. Hypothetically, farms that are vaccinating their piglets may pressurize *M. hyopneumoniae* to develop a higher genetic diversity, directly or indirectly influencing effects at the antigenic level. Antigenic variation is thought to help the bacterium to persist in its host by evading the immune response (Assunção et al., 2005; de Castro et al., 2006). On the other hand less variation may as well be a consequence. In farms where strains are present which are closely related to the vaccine strain, vaccination might suppress these strains, which might result in a lower degree of diversity. Clearly, the effect of vaccination on genetic and antigenic diversity of *M. hyopneumoniae* strains remains to be elucidated (Simionatto et al., 2013) and further research is necessary, e.g. by examining the diversity in vaccinated and non-vaccinated batches within a herd.

Other herd and batch factors, and most probably a combination of these factors, might have influenced the high diversity seen in the farms in the study in chapter 3.1. For studying the possible impact of the number of circulating *M. hyopneumoniae* strains per batch on the presence and severity of lung lesions, we also collected data on herd characteristics considered to be risk factors affecting the severity of respiratory disease in general. This was done to correct for confounding factors. However no direct associations were studied between these herd characteristics and the *M. hyopneumoniae* diversity at herd level as this was not the main objective of the study. More research is thus needed to clarify these factors.

In general, most studies showing a rather limited *M. hyopneumoniae* diversity at herd level, examined a limited number of herds (three or four) (Pantoja et al., 2016; Takeuti et al., 2017; Vranckx et al., 2011; Vranckx et al., 2012b). A high *M. hyopneumoniae* diversity was observed when ≥ 5 herds were examined: 109 herds (Nathues et al., 2011), 48 herds (Charlebois et al., 2014), 5 herds (Burgher Pulgarón et al., 2015) and in the chapter 3.1 ten herds. The reason for this finding is unclear. Most probably, when investigating a higher number of herds, more pigs from different origins are investigated and the probability to detect different *M. hyopneumoniae* is higher.

In the study in chapter 3.1 we demonstrated that in 102 lungs two different *M. hyopneumoniae* strains were detected. This corroborated previous results of Vranckx et al., (2011) and Nathues et al. (2011). In our study, in lung washings from six pigs, three different strains were detected. To date MLVA is able to detect up to at least three strains in one sample provided that the difference in concentration between the strains in the sample is less than tenfold (Vranckx et al., 2011). However, it is possible that even more *M. hyopneumoniae* strains might have been present within one pig.

### 4.1.2. Impact of the genetic diversity of *Mycoplasma hyopneumoniae* on the severity and prevalence of lung lesions

A possible link between the presence of multiple subsequent or simultaneous infections with different *M. hyopneumoniae* strains and the presence or severity of *M. hyopneumoniae*-like lesions had been suggested before the start of this thesis (Villarreal et al., 2009; Vranckx et al., 2011). However, Charlebois et al. (2014) could not

confirm this, when looking at individual lung level. Many herd characteristics possibly influencing the severity and/or prevalence of lung lesions were included into the statistical model in the study in chapter 3.1. The results in chapter 3.1 showed that the prevalence and severity of *M. hyopneumoniae*-like lesions were higher when batches of pigs were infected with different *M. hyopneumoniae* strains. The exact mechanisms leading to this phenomenon remains to be elucidated. For instance different strains might stimulate the immune system of the host to a different extent, resulting in a prolonged inflammatory reaction. However, in order to solve this question, more insight in the pathogenesis and potential virulence factors of *M. hyopneumoniae* would be helpful.

### 4.1.3. Use of MLVA in the field

The VNTR (Variable Number of Tandem Repeat) typing appeared to be very useful to assess strain diversity of *M. hyopneumoniae* in BALF collected from slaughter lungs. Previous studies reported that the technique was also useful to investigate the molecular epidemiology of *M. hyopneumoniae* outbreaks (Dos Santos et al., 2015; Mayor et al., 2007). The VNTR technique is relatively inexpensive compared for instance to next generation sequencing, although the costs depend on the numbers of VNTR amplified. No prior isolation of the *M. hyopneumoniae* strains is required, which makes the technique more accessible for laboratories not specialised in *M. hyopneumoniae* isolation. The multiplex PCR is easy to perform, but the gel or capillary electrophoresis and especially the interpretation of the obtained electropherograms require specialised knowledge. The latter is most probably the reason why to date the technique is not available as a routine diagnostic tool. The most suitable samples to perform MLVA are tracheobronchial swabs or BALF from live animals. In dead animals, lung washings at the main bronchi, are also suitable. Although nasal swabs are easy to obtain, they are considered less suitable for MLVA. They might contain too many inhibiting factors and the detection limit of MLVA is 100 *M. hyopneumoniae* organisms per µl DNA extract (Vranckx et al., 2012a; Vranckx et al., 2011; Vranckx et al., 2012b). It is well known that higher amounts of *M. hyopneumoniae*-DNA are detected closer to the *M. hyopneumoniae* multiplication sites namely the trachea-bronchial sites compared to the nasal cavities (Fablet et al., 2010).

The results in chapter 3.1 imply that external biosecurity measures to avoid introduction of new strains in the herd are important to reduce the severity and the prevalence of the *M. hyopneumoniae* lesions. Purchase of gilts or weaned pigs from multiple sources, proximity of other pig herds or contact with pig transport to the slaughter house, especially multiple source pig transport are some points of attention that can be raised. Internal biosecurity measures, such as not mixing pigs from different ages combined with high stocking densities are important as well, as it is possible that *M. hyopneumoniae* strains from one group of pigs can be transmitted to another group of pigs, within the same herd.

## 4.2. Impact of $PM_{10}$ and $NH_3$ on lung lesions and production parameters in finishing pigs

*Mycoplasma hyopneumoniae*, PM and $NH_3$ might impair the function of the mucociliary clearance apparatus in a synergistic manner, resulting in a defect of the first line defence mechanism of the respiratory tract (Gonyou et al., 2006; Johannsen et al., 1987; Pearson and Sharples, 1995; Thacker, 2006) and PM might influence the innate

and adaptive immune response (Miyata et al., 2011). In intensive pig production systems, pigs are chronically exposed to environmental stressors (Done, 1991). However, no long-term studies determining the simultaneous impact of PM and $NH_3$ on production parameters and respiratory health in finishing pigs were available at the start of this PhD. In addition, no agreement in existing literature on the separate effects of PM or $NH_3$ on these parameters was obtained. Therefore the study in chapter 3.2 assessed the simultaneous influence of $PM_{10}$ and $NH_3$ on production parameters and respiratory health from entrance of the pigs into the finishing unit until slaughter.

In the study in chapter 3.2 the focus was on the main aerial pollutants $PM_{10}$ and $NH_3$. $PM_{2.5}$ and $PM_1$ were measured as well during the study and associated with the outcome variables in this chapter. However these results were not included in the manuscript as no significant results were obtained.

### 4.2.1. Measured concentration of $PM_{10}$ and $NH_3$ in the finishing unit

The average $PM_{10}$ and $NH_3$ concentrations for the entire finishing period in both replicates in chapter 3.2 determined in one farm were 2,230 $\mu g/m^3$ (ranging from 1,707-3,280 $\mu g/m^3$) and 28.2 ppm (ranging from 17.2 - 38.2 ppm), respectively. Previous studies which also measured PM and/or $NH_3$ concentrations in finishing pigs are scarce. According to Takai et al. (1998), mean total PM in various sections of swine facilities in different countries was 2,190 $\mu g/m^3$ and in Van Ransbeeck et al. (2013b) under Flemish conditions concentrations ranged from 22 to 2288 $\mu g/m^3$. Explanations for the higher concentrations measured in our study might be that our PM measurements were determined at animal height in finishing pigs only, while in the study of Takai et al., (1998) the average value was obtained through sampling at different sampling points (at animal height, at human head height and at the ventilation exhaust) and different age categories were measured (sows, weaners and fatteners) in both studies. In our study the measurement equipment was placed inside the central pen in the facility with pigs inside, while in the study by Van Ransbeeck et al., (2013b), this was not the case. An empty pen was used to place the cage with the measurement equipment inside. In another study of Van Ransbeeck et al., (2013a), $NH_3$ was measured in finishing pig stables. The average $NH_3$ concentration for conventional and low emission stables were 18.7 and 16.3 ppm, respectively. These values were lower compared to the average $NH_3$ values obtained for the entire finishing period of both replicates in our study. The reason why much higher $PM_{10}$ and $NH_3$ concentrations were measured in the study in chapter 3.2 compared to the study of Van Ransbeeck et al. (2013a; 2013b) are unclear. The farm in our study had a higher number of finishing places, possibly resulting in a higher aerial pollutant concentration, as a strong correlation exists between animals/$m^3$ airspace and concentrations of airborne bacteria (Cargill et al., 2002; Wathes, 1994). No other information, such as type of feed or hygiene level in the different farms in the study by Van Ransbeeck et al. (2013a) was provided to possibly explain the lower aerial pollutant concentrations compared to our farm. Other factors which might have played a role in the high $PM_{10}$ and $NH_3$ concentrations in the farm in chapter 3.2 were a high stocking density, especially towards the end of the finishing period. Two other factors, a quick disintegration of the feed pellets caused by bad quality and automated feed delivery system implied in becoming airborne of particulates might also be taken into consideration (Aarnink and Ellen, 2007). Wet floors caused by a too short stand empty period causing the pigs to

dung on the solid floor and not on the slatted floors and bad pen hygiene related or not to the former are involved in $NH_3$ formation as manure and faeces on solid floor parts will increase $NH_3$ concentration (Aarnink and Ellen, 2007; Banhazi et al., 2008a; Banhazi et al., 2008b; Cargill et al., 1998; Cargill et al., 2002; Li et al., 1993; Wenger, 1999). Some of these factors implied in the formation of PM and/or $NH_3$ are not easily changed, for instance the stand empty period in a four week batch production system, and the automated feed delivery system. The quality of the pellets is another factor which may be difficult to influence by the farmer..

Nevertheless, it can be concluded that the $PM_{10}$ and $NH_3$ concentrations were above average in this farm. Therefore it was interesting to investigate whether they exerted an effect on production and health parameters in the pigs.

### 4.2.2. Impact of $PM_{10}$ and $NH_3$ on production and respiratory health parameters in grower-finisher pigs

No significant impact was demonstrated of $PM_{10}$ and $NH_3$ on ADG and mortality. This finding is in line with the results of Van 't Klooster et al., (1993), Janssens and Feddes (1995), and Takai et al., (1995). In Carpenter and Moulsley (1986) it was stated that if PM alone is considered, it is capable of affecting the performance only at very high levels, commonly not encountered in practice (300 $mg/m^3$). The association of $PM_{10}$ and $NH_3$ with ADG in the study in chapter 3.2, was borderline not significant (P=0.08 for both associations). Therefore it may be concluded that under the circumstances of the study in chapter 3.2, PM even in combination with $NH_3$ is not able to affect ADG. On the other hand, it may be possible that under unfavourable conditions in the field such as other (respiratory) infections next to *M. hyopneumoniae*-infections, added on top of the adverse stable climate, an effect on daily weight gain or other performance factors will be noticeable. Further research may enlighten the outcome of these complicated interactions.

The findings in chapter 3.2 indicate that $PM_{10}$, especially in the second half of the finishing period influenced the prevalence and severity of pneumonia. This might make sense as lesions which may have occurred by exposure to aerial pollutants in the first part of the finishing period might have been resolved already at the timing of slaughter. In general, *Mycoplasma*-like lesions are reported to be healed after twelve weeks (Sørensen et al., 1997) and upon healing, fissures appear and may remain for a period of twelve weeks as well (Kobish et al., 1993; Van Alstine, 2012). Associating the $PM_{10}$ and $NH_3$ concentrations with the prevalence of fissures was performed to determine the impact of these aerial pollutants on the *M. hyopneumoniae* lesions occurring earlier in the finishing period, but no significant associations were obtained. Another option to assess the association of aerial pollutants with early *M. hyopneumoniae* lesions was euthanizing pigs in earlier stages throughout the finishing period and associate the priory measured $PM_{10}$ and $NH_3$ concentrations with lung lesions at timing of necropsy. This was not performed as it requires to sacrifice healthy pigs. The $NH_3$ concentrations seemed to influence the prevalence and severity of pneumonia to a lesser extent than the $PM_{10}$ concentrations. A hypothesis might be that PM is more toxic compared to $NH_3$ alone. Next to exerting its own damage to the respiratory tract described before, PM is also able to adsorb $NH_3$ on its porous surface (Cai et al., 2006). In that way $NH_3$ is not trapped by the mucus membrane of the upper respiratory tract, but can be inhaled much deeper into the airway

where it can cause more damage (Curtis et al., 1975; Wenger, 1999). Both $PM_{10}$ and $NH_3$ measured in the second half of the finishing period and during the entire finishing period were significantly associated with the prevalence of pleurisy. When observing the multivariable associations, the only remaining significant association was $PM_{10}$ with prevalence of pleurisy. The entrance in the respiratory tract of pathogens causing pleurisy lesions such as *A. pleuropneumoniae*, *H. parasuis*, *M. hyorhinis* and *P. multocida* (Enøe et al., 2002), may have been facilitated by $PM_{10}$ influencing the function of the mucociliary apparatus. This is merely hypothetical, as no diagnostics for pathogens, other than *M. hyopneumoniae* were performed in our study. Pleurisy is an uncommon lesion in *Mycoplasma hyopneumoniae*-infections, although it has been reported (Falk and Lium, 1991; Ross, 1992). Other associations which had a tendency to be significantly associated were the $PM_{10}$ concentrations measured during the entire finishing period and $NH_3$ concentrations during the second half of the finishing period with the prevalence of *M. hyopneumoniae* positive nPCR results. Again, the high $PM_{10}$ burden in the pig facilities in the study, as demonstrated by our measurements and by comparing our results with other research, might have favoured *M. hyopneumoniae* circulation. Although it could be possible to conduct a study with a higher sample size or include more replicates to investigate these trends further, such a study will have practical implications in terms of feasibility.

### 4.2.3. Practical implications for the herd veterinarian

In those farms where vaccination against *M. hyopneumoniae* infections is applied, but which still experience major problems with *M. hyopneumoniae,* it might be valuable before pointing towards the efficacy of the used vaccine, to evaluate the stable climate, next to evaluating other factors already discussed in the introduction of this thesis. User friendly, low maintenance and long-term monitoring of air quality is possible with data loggers for temperature, relative humidity and certain gasses (CO, $CO_2$ and $NH_3$,…), however it is questionable whether all data loggers, especially in the cheaper price range will continue to function properly under the harsh conditions in a pig facility. For PM measurements, the devices are not suitable to measure continuously in a pig facility and require too much maintenance to be used for a continuous monitoring of indoor air quality by farmer or herd veterinarian (clogging of the air pump, changing of the filter daily,…). The intensive measuring strategy used in chapter 3.2 might even not be necessary for the herd veterinarian to obtain an idea of the concentrations of aerial pollutants during the finishing period. Van Ransbeeck et al. (2012) observed that the best measuring strategy for a finishing stable is to obtain PM concentrations of four to five equally distributed sampling periods of 48 h. during the finishing period. For $NH_3$ two measurement periods in the first half of the finishing period and two in the second half of the finishing period are sufficient. This allows the reconstruction of the evolution of PM and $NH_3$ concentrations of the entire finishing period without losing important data characteristics. Advices directed to the farmer on actions to reduce the PM and $NH_3$ concentration in the stable are revised in Aarnink and Ellen (2007) and Ulens et al., (2015).

From the study it can be concluded that $PM_{10}$, especially high concentrations in the second half of the finishing period, might affect the severity and prevalence of pneumonia lesions, the prevalence of pleurisy and the *M. hyopneumoniae* circulation towards the end of the finishing period.

## 4.3. Impact of Deoxinivalenol on a *Mycoplasma hyopneumoniae* infection

Antonissen et al. (2014) indicated a knowledge gap regarding the interactions between mycotoxins and infectious diseases. To the best of our knowledge, no studies were available investigating the impact of DON in-feed on *M. hyopneumoniae* infections at the start of this PhD. Part of the thesis therefore focused on investigating the impact of DON on a *M. hyopneumoniae*-infection, as it is likely that pigs under commercial husbandry circumstances are simultaneously exposed to both health hazards during their life course.

### 4.3.1. Deoxynivalenol- concentration administered to the pigs

The concentration of DON detected in feedstuffs and feed worldwide is mostly in the lower to moderate concentration range (Placinta et al., 1999; Rodrigues and Naehrer, 2012a; Streit et al., 2012). In 535 corn samples and 436 wheat/bran samples originating from Central-Europe, the average DON-concentrations of the positive samples were 1,421 μg/kg and 1,534 μg/kg, respectively (Rodrigues and Naehrer, 2012b). The level of 1,800 μg/kg in chapter 3.3 was chosen based on exceeding the maximum guidance no-effect level for feed intake in pigs according to EU regulation (900 μg/kg) and the report of Madson et al., (2014) in which it was stated that delayed or suppressed immune responses might be visible from 1,000 μg/kg onwards. It was chosen not to exceed 2,000 μg/kg-4,000 μg/kg, as a dose-related decrease in daily feed intake is observed from these concentrations onwards (Bergsjø et al., 1992; Dänicke et al., 2004; Prelusky et al., 1994).

### 4.3.2. Impact of Deoxynivalenol on the pigs and possible factors influencing Deoxynivalenol to exert its function

The DON inoculation procedure of the feed was already successfully used in pigs in a study of Goossens et al. (2012) where it was investigated if DON affects the oral bioavailability of frequently used antibiotics in pigs. Before the start of the study, no *in vitro* studies were performed to examine the possible effect of DON on *M. hyopneumoniae*. A study using tracheal explants and DON influencing the attachment of *M. hyopneumoniae* could be considered, as attachment is the first step in causing disease. On the other hand, *M. hyopneumoniae* is localised superficially at the cilia of the upper respiratory tract. Although it is known that DON may reach the lung in pigs, the mycotoxin might not reach that site of the respiratory tract. From the point of view of immune modulation, DON could also affect the pathogenesis of *M. hyopneumoniae*, which is very interesting as it is known that *M. hyopneumoniae* is able to modulate the innate and adaptive immune response. However, as the pathogenesis of *M. hyopneumoniae* is not yet fully elucidated, this would be difficult to test *in vitro*, also because *M. hyopneumoniae* grows very slowly.

The DON inoculation medium in the feed in our study was thoroughly mixed, as evidenced by the measured DON concentration (1,514 μg/kg) which only slightly deviated from the target DON concentration after the mixing procedure. Another approach would have been to house the pigs separately and add the DON-inoculated feed in an individual manner daily. This would ensure that each pig ingested the same amount of feed and thus approximately the same amount of DON, provided that the pigs consumed their portion of feed. The number of

HEPA-filtered units in the research facility are limited, and follow up of each pig would have been more laborious to perform, compared to the study design we performed. This would have minimised the number of animals used in the study decreasing the statistical power of the study to detect significant differences between groups.

Possible explanations why no visible effect of the DON administration to the pigs was noticed in our study will be discussed in following sections.

The level from when onwards clinical effects of DON are visible might depend on the pigs used in the study. There is a need to investigate the influence of age of the pigs on the effects of DON and possibly adapt the EU guidance value according to the age group (piglets, nursery pigs, growers, finishers, adults). Weanling mice are more prone to the effects of DON compared to adult mice (Pestka, 2008). The reports, in which age and the effects of DON in pigs are compared are scarce. Tiemann and Dänicke (2007) observed that prepuberal gilts react more sensitively to DON-contaminated diet compared to pregnant sows. For instance, histopathological changes such as glycogen decrease and interlobular collagen uptake were only observed in prepuberal gilts. Other factors may also influence the clinical outcome of consuming DON-contaminated feed such as gender/hormonal status, health status, nutritional balance, genetics or combinations of these factors (Dersjant-Li et al., 2003).

An adaptive response or tolerance to the effects of DON in pigs has been observed after DON-administration of one week. However, no further explanation is given for this observed effect, other than the possible rapid clearance of DON from the body of the pigs (Foster et al., 1986; Friend et al., 1986; Pollmann et al., 1985). As the pigs in the study in chapter 3.3 were fed the DON-contaminated feed for five weeks, it is a possibility that the pigs were adapted to the effects of DON after one week of exposure. Another possible pathway for reduced effects of supplemented DON in-feed is detoxification of DON, which is possible by bacterial transformation from DON to metabolites with reduced toxicity. In pigs only a moderate transformation of DON to de-epoxy-DON occurs in the hind gut (Dänicke et al., 2003).

In our study, the pigs were only exposed to one mycotoxin (DON), whereas in the field pigs are likely exposed to different mycotoxins simultaneously, which may enhance the toxic effect (D'Mello et al., 1999). In pigs, the combination of DON and fusaric acid (Smith et al., 1997) and DON and FB1 (Harvey et al., 1996) resulted in synergistic effects.

In the study in chapter 3.3 the pigs were fed an artificial DON-spiked diet. Before spiking the diet with DON, it was tested for the presence for other *Fusarium* trichotecenes and zearalenone. Feed naturally contaminated with *Fusarium* species, seems to have a more pronounced effect on different parameters, such as feed intake and weight gain versus feed artificially contaminated with mycotoxins (Forsyth et al., 1977; Foster et al., 1986; Rotter et al., 1994; Trenholm et al., 1994). The reason why this occurs is not known. Some authors hypothesise that other unidentified mycotoxins, toxic metabolites or substances, such as bacterial polysaccharide or metabolites produced by the plant host, may affect the toxicity of trichothecenes (EFSA, 2007; Foster et al., 1986; Rotter, 1996; Trenholm et al., 1994). In our study, 3ADON and 15ADON were included in the inoculum to prepare the DON-spiked feed. Although Pestka et al. (1987) found that the emetic activity of 15ADON in swine in a dose range of 25-200 μg/kg was very similar to that of DON, the toxicological relevance of the acetyl derivatives has

been underestimated due to lack of information on toxicity and toxicokinetics (Broekaert et al., 2015). In a Dutch field investigation, both ADONs were detected in 21 and 7% of the maize samples, more frequently than DON itself (Van Asselt et al., 2012). In a study of Broekaert et al. (2015) it was demonstrated that in pigs the absorbed fraction after oral administration was 100% in case of DON, but as well for both ADONs. Broekaert et al. (2015) answered the key question regarding the in vivo hydrolysis of 3ADON and 15 ADON to DON in pigs: 3ADON and 15ADON were completely hydrolysed presystemically, possibly indicating an underestimation of the degree of contamination and the toxicity of DON.

Masked mycotoxins may be present in naturally contaminated feed. These substances will usually not be detected. In plants, glycosylation is an important defence mechanism against xenobiotics, including mycotoxins (Michlmayr et al., 2015). Deoxynivalenol can be glycosylated and stored in plant vacuoles. From these masked mycotoxins, the precursor mycotoxin, DON, can be reactivated by hydrolysis when digested. Nagl et al. (2014) indeed showed that Deoxynivalenol-3-glucoside (D3G) is nearly completely hydrolysed in the intestinal tract of the pigs.

It might be possible that DON under field circumstances has a more pronounced effect in *M. hyopneumoniae* infected pigs compared to our findings described in chapter 3.3. Intake of DON may affect the acquired immunity response upon vaccination against *M. hyopneumoniae*. ØVernes et al. (1997) found a dose dependent decrease in secondary antibody response to tetanus toxoid in pigs when pigs were fed a DON-spiked diet. In Savard et al. (2015), a reduced efficacy of a modified live vaccine against PRRSv was observed in pigs fed a DON-spiked diet. Pigs fed a diet containing DON or Fumonisin B1 (FB1) showed a reduced anti-ovalbumin antibody production with a decreased lymphocyte proliferation (Grenier et al., 2011).

No reports investigating the impact of DON-ingestion in pigs on *M. hyponeumoniae* vaccine efficacy are available yet. However, a decreased specific antibody response after vaccination with formol inactivated *Mycoplasma agalactiae* in pigs was observed after ingestion of low doses of FB1 (Taranu et al., 2005).

### 4.3.3. Impact of DON on a *Mycoplasma hyopneumoniae* infection

From the results in the study in chapter 3.3, it can be concluded that under the circumstances of this experiment, DON did not influence the severity of a *M. hyopneumoniae* infection. After absorption in the gut, DON reaches primarily the most perfused organs, such as the myocardial muscle, kidneys, brain and the lung (Goyarts and Dänicke, 2006; Prelusky and Trenholm, 1991). As *M. hyopneumoniae* does not invade the lung parenchyma and is merely attached superficially to the cilia of the upper respiratory tract (Blanchard et al., 1992; Jaques et al., 1992; Maes et al., 2017), it is possible that mycotoxin concentrations were too low to affect the *M. hyopneumoniae* infection. Remarkably, in mice, the effects of intranasal exposure of DON compared to oral distribution resulted in 1.5-3 times higher concentrations of DON in plasma and tissue. The induction of pro-inflammatory cytokines (IL1-β, IL-6 and TNF-α) in several tissues including the lung, was higher after intranasal compared to orally application of DON (Amuzie et al., 2008). No such studies are available in pigs. It cannot be ruled out that mycotoxins can act as indoor air contaminants. Feed contaminated with DON can generate PM that can be inhaled by the pigs (Jarvis and Miller, 2005). This route might in theory constitute an additional burden for the

pig under field circumstances, next to oral intake of DON, although it is stated by Jarvis and Miller (2005) that this is very unlikely for this mycotoxin.

## 4.4. Effect of vaccination with a commercial bacterin on a *Mycoplasma hyopneumoniae* infection

### 4.4.1. Characteristics of the commercial bacterin used in chapter 3.4

The high heterogeneity seen in *M. hyopneumoniae* strains might be a possible explanation for the variable results obtained with commercial vaccines at herd level. Charlebois et al. (2014) indeed demonstrated that almost half of the *M. hyopneumoniae* isolates from slaughter lungs in Canada presented less than 55% genetic homology with a selected J-strain based vaccine and other reference strains, among which USA232. On the other hand, Villarreal et al. (2012) did not detect significant differences in protective efficacy against an experimental *M. hyopneumoniae* infection when animals were vaccinated with an experimental vaccine containing the challenge strain or a commercial bacterin based on the J-strain. The antigen concentration included in the experimental bacterin might however have been lower compared to commercial bacterin. The *M. hyopneumoniae* vaccine strain BA2940-99, used in chapter 3.4 from this thesis, was isolated in 1999 in the USA from a pig with enzootic pneumonia and genetic and proteomic differences between this strain and J-strain were detected. This vaccine was very efficacious against a challenge with two *M. hyopneumoniae* strains, the highly virulent F7.2C and the low virulent F1.12A, isolated in Belgium, in 2000 and 1999, respectively. Unfortunately, studies comparing genetic and proteomic relatedness between the vaccine strain and our challenge strains are not available. Obviously the importance of the *M. hyopneumoniae* strain included in the vaccine for induction of protection warrants further investigation.

The impact of the adjuvant on the efficacy of a vaccine should as well not be underestimated. Adjuvants are added to the vaccine to augment the adaptive immune response, to direct the type of adaptive immune response in order to obtain the most effective forms of immunity for the specific pathogen, to generate effective immunological memory and may decrease the amount of antigen needed in the vaccine (Coffman et al., 2010; McKee et al., 2007; Spickler and Roth, 2003). The vaccine used in chapter 3.4 is a detoxified *Escherichia coli* lipopolysaccharide (LPS) oil-in-water emulsion adjuvanted bacterin, with thiomersal as excipient (Product Leaflet Hyogen). Cell wall peptidoglycan or lipopolysaccharide obtained from Gram negative bacteria as (part of the) adjuvant, enhance the immune response against less immunogenic co-administrated antigens. This adjuvant activity is mediated through activation of Toll-like receptors (TLR-4), stimulating both cellular and humoral immune responses (Petrovsky and Aguilar, 2004). Oil-in-water emulsions contain microdroplets of oil in water. They release the antigen fairly quickly, but the droplets of oil may be able to carry antigens to the lymph nodes and antigen depots may be formed on antigen presenting cells in the lymph nodes rather than at the reaction site (Spickler and Roth, 2003). Oil emulsion adjuvants may be more efficacious compared to the aluminium hydroxide adjuvanted *M. hyopneumoniae* vaccines (Spickler and Roth, 2003). The downside is that these oil-based adjuvants are known to cause more injection-site reactions. Water-in-oil type emulsions may possibly induce

necro-ulcerative lesions at the injection site. Oil-in-water type emulsions on the other hand are generally well-tolerated (Jansen et al., 2006). In the study performed in chapter 3.4 no macroscopically visible injection-site reactions were noticed.

### 4.4.2. Efficacy of the commercial bacterin used in chapter 3.4

To mimic the field situation, it was decided to challenge the pigs with two contemporary genetically different *M. hyopneumoniae* strains. As shown in chapter 3.1 multiple strains are circulating in a herd and pigs can be infected with two or even three genetically different *M. hyopneumoniae* strains. The exact mechanism of multiple strains interacting in the pig is not known, a more pronounced inflammatory reaction might be involved causing more lung lesions. At the start of this thesis no information was available regarding the effect of vaccination when challenging the pigs with two genetically different *M. hyopneumoniae* strains. Both strains had been characterized at genomic and proteomic level (Calus et al., 2007; Stakenborg et al., 2006; Vranckx et al., 2011). Under the conditions of an experimental challenge, it might be expected that a higher infection dose than encountered in the field is used (Meyns et al., 2004; Villarreal et al., 2009). Even under these challenge conditions, the vaccine was very efficacious, as shown by significantly reduced disease signs, lung lesions and *M. hyopneumoniae* multiplication in the lungs of vaccinated pigs, compared to non-vaccinated animals.

It was described that increased pro-inflammatory cytokines IL-1β and IL-6 levels in BALF of *M. hyopneumoniae* challenged pigs were associated with the development of pneumonia lesions (Ahn et al., 2009; Asai et al., 1994; Asai et al., 1993; Thanawongnuwech et al., 2004). In our study, IL-1β and IL-6 antibody levels in BALF were indeed significantly lower in the vaccinated challenged pigs compared to the non-vaccinated challenged pigs and did not statistically differ from those found in the non-challenged pigs. Although TNF-α levels have also been associated with severity of lung lesions, no effect of vaccination on this parameter was detected here.

Although the vaccine was very efficacious to reduce the severity of lung lesions and clinical signs under these experimental conditions, confirmation of our results in field studies is desirable. Under field conditions, other respiratory pathogens are circulating, possibly challenging the immune system of the pigs or aggravating lung lesions and stable climate, housing and management conditions are mostly not optimal.

## General conclusions

From the studies performed in this thesis, it can be concluded that both number of different *M. hyopneumoniae* strains and air quality, more specifically PM10 and $NH_3$, may play a role in the severity or prevalence of the *M. hyopneumoniae* lesions. Deoxynivalenol under the circumstances of an experimental *M. hyopneumoniae*-infection, seemed to play a less significant role and the commercially available *M. hyopneumoniae* bacterin was able to significantly reduce the severity of lung lesions and clinical signs in an experimental *M. hyopneumoniae* challenge infection with two genetically different strains.

More specifically it can be concluded that:

- Many different *M. hyopneumoniae* strains were circulating within and between several slaughter batches from closed pig farms in Flanders, practising vaccination against *M. hyopneumoniae* in their piglets
- In slaughter batches where more than one *M. hyopneumoniae* strain was detected, more severe pneumonia lesions and a higher prevalence of pneumonia was observed
- Pigs may be simultaneously infected with two and even three genetically different *M. hyopneumoniae* strains
- Under the circumstances of this research $PM_{10}$ and $NH_3$ did not significantly affect production parameters mortality and ADG. $PM_{10}$ and $NH_3$ numerically decreased ADG in the finishing pigs (borderline not significant)
- The respiratory health of finishing pigs was significantly affected by $PM_{10}$: an increasing $PM_{10}$ concentration measured during the entire fattening period resulted in a higher prevalence of pleurisy in the pigs
- The effect of $PM_{10}$ on prevalence and severity of pneumonia was more pronounced than the effect of $NH_3$
- $PM_{10}$ tended to be associated with the presence of *M. hyopneumoniae* as detected by nPCR in nasal swabs of the finishing pigs
- Deoxynivalenol administration in the feed at two times the EU recommended maximum level (1,800 µg/kg) did not aggravate the severity of an experimental *M. hyopneumoniae* infection
- A commercial bacterin based on strain BA2940-99 and adjuvanted with non-toxic *Escherichia coli* LPS oil-in –water emulsion was very efficacious in reducing the clinical parameters and pathological parameters of an experimental challenge infection with two genetically different *M. hyopneumoniae* strains on two consecutive days
- The commercial vaccine was able to reduce but not prevent *M. hyopneumoniae* infection with two genetically different *M. hyopneumoniae* strains

## Future research

From the present thesis, some items for future *M. hyopneumoniae* research may be proposed:

It is not clear why batches of pigs infected with multiple *M. hypopneumoniae* strains have more severe lung lesions compared to when only a single strain is present. Further elucidating the pathogenesis of *M. hyopneumoniae* infections and identifying possible virulence factors of this bacterium might be helpful to obtain better insights in this matter. Furthermore, there is a need to investigate the dynamics and the stability of *M. hyopneumoniae* strains in a herd, the transmission of *M. hyopneumoniae* strains from sow to piglets and the triggering factors for *M. hypopneumoniae* strain diversity to occur in the herd. The impact of vaccination strategies, other interventions or herd practices on *M. hyopneumoniae* strain diversity are not yet known.

Further research could elaborate on the impact of PM10, other PM fractions $PM_{2.5}$ and $PM_1$ and $NH_3$ on *M. hyopneumoniae* infections, e.g. by monitoring different successive batches and/or by euthanizing a representative number of pigs throughout the finishing period to observe the impact of aerial pollutants in the first weeks of the finishing period. It would be interesting to investigate the impact of aerial pollutants in piglets or nursery pigs and the consequences for the production parameters and respiratory health in the finishing period thereafter. The impact of maximum measured PM concentrations on respiratory disease (coughing, nPCR positivity for *M. hyopneumoniae*, lung lesions,…) could be determined. Furthermore, the impact of different mitigation strategies on the indoor aerial pollutant concentrations inside pig facilities could be investigated more into depth, more specifically the impact on (respiratory) health and production in different age categories of pigs.
Also, further research should be done on the possible effects of DON on pig health in general and a *M. hyopneumoniae* infection more specifically. These factors include: natural vs. artificial contamination, tolerance of the pigs, genetic factors, nutritional status, hormonal balance and age of the pigs, as well as multi-mycotoxin exposure. More research is also warranted to assess the impact of DON and mycotoxins in general on vaccine efficacy in *M. hyopneumoniae* vaccinated pigs.

The possible influence of different vaccine strain on protection induced by bacterins against infection with *M. hyopneumoniae* field strains also requires further studies. A deeper investigation regarding protection conferred by vaccination with homologous/heterologous strains is necessary. The relative importance of the vaccine strain versus the used adjuvant in the vaccine should be investigated further.

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SUMMARY

# *Summary*

# SUMMARY

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary agent in enzootic pneumonia and plays a key role in the porcine respiratory disease complex. Infections with *M. hyopneumoniae* occur worldwide and negatively impact growth and feed efficiency, increase antimicrobial use, and causes infections with other respiratory agents being facilitated. It is known that the outcome of a *M. hyopneumoniae* infection can be quite variable, ranging from subclinical infections to serious clinical cases, causing tremendous losses. Many factors involved in the severity of a *M. hyopneumoniae* infection are known. Such factors are management practices, interactions with other bacterial or viral respiratory agents, antimicrobial therapy and vaccination strategies. Other factors such as *M. hyopneumoniae* diversity, concentration of toxic air pollutants in the stable, and mycotoxins in the feed are less well studied, and the impact on *M. hyopneumoniae* infections are less well known. As *M. hyopneumoniae* is still causing economic losses worldwide, research is also aimed at developing new vaccines to control the disease in a better way.

The general aim of this thesis was to investigate factors that influence the occurrence of pneumonia lesions and *M. hyopneumoniae* infections in order to achieve a better control of the disease.

The specific objectives were:

1. To determine the diversity of *M. hyopneumoniae* in Flemish pig farms and to investigate its impact on lung lesions at slaughter
2. To investigate the impact of particulate matter and ammonia levels in the stable on pneumonia and pleurisy lesions
3. To assess the impact of deoxynivalenol and acetyl-derivates in the feed on an experimental *M. hyponeumoniae* infection
4. To investigate the effect of vaccination with a commercial vaccine on an experimental *M. hyopneumoniae* infection with two genetically different *M. hyopneumoniae* strains

First, in the general introduction a summary of the literature is provided starting with describing how the severity of *M. hyopneumoniae* infections can be determined. This can be performed by scoring the pigs in a clinical way with a respiratory disease score. Next, the different scoring systems to determine the severity of macroscopical and microsocopical *M. hyopneumoniae* lesions are described. This part is concluded with determining the number of *M. hyopneumoniae* organisms in the respiratory tract. Subsequently all possible factors influencing the severity of *M. hyopneumoniae* infections are summarized.

In the first study in chapter 3.1 *M. hyopneumoniae* diversity in three batches of slaughter pigs originating from ten closed farms or closed production systems, vaccinating their piglets against *M. hyopneumoniae* was investigated with Multiple Locus Variable Number of Tandem Repeats (MLVA). The impact of the number of strains detected in these batches on the prevalence and severity of M. *hyopneumoniae* lung lesions was determined, taking into account several important risk factors at herd level for respiratory disease. A very high *M. hyopneumoniae* genetic diversity was observed in our study. In total, 135 genetically different *M. hyopneumoniae* strains were detected in the entire dataset from 600 bronchoalveolar lavage fluids collected at slaughter. The average number

of different strains per batch was seven with a minimum of one and a maximum of 13 different *M. hyopneumoniae* strains. In 102 lungs, two and in six lungs, three genetically different *M. hyopneumoniae* strains were detected. In batches where more than one different *M. hyopneumoniae* strain was detected, more severe and a higher prevalence of *Mycoplasma*-like lesisons, and a higher prevalence of fissures was observed. These results imply that MLVA-testing revealed a high genetic *M. hyopneumoniae* diversity in batches of Flemish slaughter pigs and that *Mycoplasma*-like lung lesions were more severe and a higher prevalence of pneumonia and fissures was detected when a higher *M. hyopneumoniae* diversity is observerd in a batch of slaughter pigs.

In chapter 3.2 the simultaneous and long-term impact of $PM_{10}$ (ranging from 1,707-3,820 μg/m$^3$) and ammonia ($NH_3$) (ranging from 17.2-38.2 ppm) on productions parameters (daily weight gain and mortality), respiratory health and the presence of *M. hyopneumoniae* in finishing pigs was investigated. For this study a closed pig herd experiencing clinical problems of *M. hyopneumoniae* was selected. In total, 1095 finishing pigs were included in the study. The $PM_{10}$ concentrations were measured continuously and the $NH_3$ concentrations semi-continuously over the entire finishing period. The $PM_{10}$ and $NH_3$ concentrations did not seem to affect the performance parameters mortality and ADG under these circumstances. Increasing $PM_{10}$ concentrations influenced the prevalence of pleurisy. When $PM_{10}$ was considered in the univariable models it seemed to influence the prevalence of pneumonia and pleurisy more than $NH_3$ in the second half of the finishing period. This was also observed for the severity of *Mycoplasma*-like lesions (entire finishing period and second half of the finishing period). In the univariable models $PM_{10}$ and $NH_3$ seemed to increase the number of *M. hyopneumoniae* nPCR positive pigs, however in the multivariable associations, this effect was reduced to non-significance. These findings indicate that respiratory health was influenced by increasing $PM_{10}$ concentrations.

In chapter 3.3 the clinical impact of a moderately high deoxynivalenol (DON)-concentration for five weeks administered in feed to weaned piglets was determined. The DON-concentration was aimed at two times the maximum guidance level for DON in pig feed by the European Commission (1,800 μg/kg). Although the *M. hyopneumoniae* experimental inoculations were successful, no effect of DON-ingestion in the *M. hyopneumoniae* challenged pigs were observed.

In chapter 3.4, the efficacy of vaccination against experimental infection with two genetically different *M. hyopneumoniae* strains in weaned piglets was determined. A commercial available vaccine was used. This vaccine, is a whole cell bacterin, using *M. hyopneumoniae* strain 2940, isolated in 1999. It is adjuvanted with a detoxified *Escherichia coli* lipopolysaccharide (LPS) oil-in-water emulsion and thiomersal as excipient. The *M. hyopneumoniae* challenge infections were performed with two genetically different *M. hyopneumoniae* strains as in chapter 3.3. The vaccine was very efficacious against the experimental challenge: the vaccinated piglets coughed significantly less, significantly less macroscopic lung lesions were detected, a significant lower amount of *M. hyopneumoniae*-DNA and IL-1ß and IL-6 concentrations in the BALF was detected in the non-vaccinated, challenged pigs.

From the studies performed in this thesis it can be concluded that a very high *M. hyopneumoniae* diversity is present in batches of Flemish slaughter pigs and that a higher prevalence of pneumonia and fissures and more severe *Mycoplasma*-like lung lesions are observed in batches of slaughter pigs were more than one different *M.*

*hyopneumoniae* strain is detected. The respiratory health of the pig was more influenced by $PM_{10}$ compared to $NH_3$. Under the circumstances of an experimental study DON in a concentration of 1,800 μg/kg did not influence the severity of an *M. hyopneumoniae* infection. The tested commercial bacterin was very efficacious against an experimental challenge with two contemporary and genetically different *M. hyopneumoniae* strains. Nevertheless, the vaccine was not able to prevent the pigs from being colonized.

Future research may focus on the triggering factors for *M. hyopneumoniae* diversity to arise and why $PM_{10}$ seems to influence respiratory health more compared to $NH_3$. Other interesting pathways to investigate further are the effects of DON on pig health in general and on *M. hyopneumoniae* infections under field circumstances, as multi-pathogen and multi-mycotoxin factors may influence these interactions. New vaccines to provide an even higher protection against *M. hyopneumoniae* may be developed, however, elucidating the pathogenesis of *M. hyopneumoniae* may be a necessity to improve this further.

SAMENVATTING

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*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is het primair agens van enzoötische pneumonie en speelt een belangrijke rol in het porcien respiratoir ziekte complex. Infecties met *M. hyopneumoniae* komen wereldwijd voor en hebben een nefaste invloed op de dagelijkse groei en voeder efficiëntie van geïnfecteerde varkens. Deze infecties brengen een verhoogd antibiotica gebruik met zich mee en infecties met andere ademhalingspathogenen vinden gemakkelijker plaats. Het is reeds lang gekend dat het resultaat van een *M. hyopneumoniae*-infectie erg variabel kan zijn: een spectrum gaande van erg milde subklinische tot zeer ernstige klinische infecties wordt aangetroffen. Deze laatste kunnen hoge economische verliezen teweeg brengen. Vele factoren betrokken bij de ernst van een *M. hyopneumoniae* infectie zijn gekend. Deze factoren zijn management, interacties met andere bacteriële en virale respiratoire agentia, gebruik van antimicrobiële middelen en het toepassen van vaccinatie. Andere factoren zoals *M. hyopneumoniae* diversiteit, toxische componenten in de stallucht en mycotoxines in het voeder werden nog minder goed onderzocht en hun impact op *M. hyopneumoniae*-infecties is dan ook minder gekend. Net omdat *M. hyopneumoniae* nog steeds veel economische verliezen met zich mee brengt, en dit op wereldwijd vlak, wordt er nog veel onderzoek uitgevoerd, gericht op het ontwikkelen van nieuwe vaccins in de hoop de ziekte beter onder controle te kunnen houden.

De algemene doelstelling van deze thesis was onderzoek uitvoeren naar factoren die het voorkomen van longletsels kunnen beïnvloeden en factoren die een invloed kunnen uitoefenen op het voorkmen van *M. hyopneumoniae*-infecties om zodoende een betere basis te leggen voor verder onderzoek om de ziekte onder controle te krijgen.

De specifieke doelstellingen waren:

1. Het nagaan van de *M. hyopneumoniae* diversiteit in Vlaamse varkensbedrijven en de impact hiervan op longletsels gedetecteerd aan de slachtlijn onderzoeken
2. De invloed van stof en ammoniak concentraties in de stal op pneumonie en pleuritis laesies nagaan
3. De invloed van deoxynivalenol en acetyl-derivaten hiervan in het voeder op een experimentele *M. hyopneumoniae*-infetie nagaan.
4. Het effect van vaccinatie met een commercieel vaccin op een experimentele *M. hyopneumoniae* infectie nagaan met twee genetisch verschillende *M. hyopneumoniae* stammen

In de algemene inleiding werd er een overzicht gegeven van hoe de ernst van *M. hyopneumoniae* letsels kan worden nagegaan. Dit kan door de dieren klinisch te scoren op de aanwezigheid van hoest. Vervolgens werden de verschillende systemen om macroscopische en microscopische longlaesies te wijten aan *M. hyopneumoniae* te scoren beschreven. Dit hoofdstuk werd afgesloten met een beschrijving van hoe het aantal *M. hyopneumoniae* kiemen in het respiratoir stelsel kan bepaald worden. Vervolgens werden alle factoren die potentieel een invloed kunnen uitoefenen op de ernst van een *M. hyopneumoniae*-infectie besproken.

In de studie in hoofdstuk 3.1 werd de *M. hyopneumoniae* diversiteit door middel van Multiple Locus Variable Number of Tandem Repeats (MLVA) in drie loten slachtvarkens afkomstig van tien gesloten bedrijven of

productiesystemen, waar de biggen gevaccineerd werden tegen *M. hyopneumoniae,* bepaald. Eveneens werd de impact van het aantal stammen op de prevalentie en ernst van de *M. hyopneumoniae* laesies bepaald, waarbij in de associaties rekening werd gehouden met allerlei factoren op bedrijfsniveau die het voorkomen van respiratoire aandoeningen kunnen beïnvloeden. Een erg hoge *M. hyopneumoniae* diversiteit werd gedetecteerd in onze studie. In de volledige dataset werden er van 600 longspoelsels, 135 verschillende *M. hyopneumoniae* stammen gedetecteerd. Het gemiddeld aantal verschillende stammen per slachtlot was zeven met een minimum van één en een maximum van 13 verschillende *M. hyopneumoniae* stammen. In 102 longen werden er twee verschillende *M. hyopneumoniae* stammen aangetroffen en in zes longen drie verschillende *M. hyopneumoniae* stammen. In loten slachtvarkens waar meer dan één verschillende *M. hyopneumoniae* stam werd aangetroffen was er een hogere prevalentie van *Mycoplasma* laesies. De letsels waren bovendien ernstiger. Eveneens werd er een hogere prevalentie van fissuren opgemerkt. Deze resultaten tonen aan dat door middel van MLVA een hoge genetische *M. hyopneumoniae* diversiteit werd aangetroffen in loten slachtvarken afkomstig van Vlaamse bedrijven. Eveneens waren de letsels ernstiger en kwamen er meer letsels voor in geval meerdere *M. hyopneumoniae* stammen werden gedetecteerd in een lot slachtvarkens.

In hoofdstuk 3.2 werd de langdurige en simultane invloed van stof $PM_{10}$ (variërend van 1,707-3,820 $\mu g/m^3$) en ammoniak ($NH_3$) (variërend van 17.2-38.2 ppm) nagegaan op productieparameters (dagelijkse groei en sterfte), respiratoire gezondheid en aanwezigheid van *M. hyopneumoniae* bij vleesvarkens. Voor deze studie werd een gesloten varkensbedrijf geselecteerd waar er klinische *M. hyopneumoniae* problemen werden geconstateerd. In totaal werden er 1095 varkens opgevolgd. De $PM_{10}$ en $NH_3$ concentraties werden respectievelijk continu en semi-continu opgevolgd gedurende de volledige mestperiode. De $PM_{10}$ en $NH_3$ concentraties hadden geen invloed op sterfte en dagelijkse groei onder de omstandigheden van de studie. Stijgende $PM_{10}$ concentraties bleken de prevalentie aan pleuritis te beïnvloeden. Wanneer enkel naar $PM_{10}$ werd gekeken in de univariabele modellen, bleek deze een grotere invloed uit te oefenen op de pneumonie en pleuritis prevalentie dan $NH_3$ in de tweede helft van de mestronde. Dit werd ook vastgesteld voor de ernst van *Mycoplasma* laesies (volledige en tweede helft mestronde). In het univariabele model bleken $PM_{10}$ en $NH_3$ beiden een invloed uit te oefenen op het aantal *M. hyopneumoniae* kiemen gedetecteerd met nPCR. In de multivariable modellen, werd deze associatie herleid tot non-significantie. De bevindingen van deze studie geven aan dat de respiratoire gezondheid beïnvloed wordt door stijgende $PM_{10}$ concentraties.

In hoofdstuk 3.3 werd de klinische impact van een matig hoge DON-concentratie nagegaan bij toediening aan gespeende biggen voor een periode van vijf weken. De concentratie aan DON in het voeder was twee maal de aanbevolen maximum grens voor DON in het voeder voor varkens (Europese Commissie). Alhoewel de experimentele *M. hyopneumoniae*-inoculaties successvol waren, werd er geen effect gezien van DON op de ernst van de *M. hyopneumoniae*-infectie.

In hoofdstuk 3.4 werd de doeltreffendheid van vaccinatie op een experimentele *M. hyopneumoniae* infectie met twee genetisch verschillende *M. hyopneumoniae*-stammen nagegaan in gespeende biggen. Het vaccin dat werd aangewend is een commercieel beschikbaar vaccin, bestaande uit een whole-cell bacterin van *M. hyopneumoniae*

stam 2940, geïsoleerd in het jaar 1999. Als adjuvant bevat het vaccin een niet-toxisch *Escherichia coli* lipopolysaccharide (LPS) olie in water emulsie met thiomersal als hulpstof. De *M. hyopneumoniae* challenge infectie werd met twee genetisch verschillende *M. hyoopneumoniae* stammen uitgevoerd (dezelfde stammen als in hoofdstuk 3.3). Het vaccin bleek zeer goed werkzaam tegen deze challenge: de gevaccineerde dieren hoestten significant minder, vertoonden significant minder longlaesies, een lagere hoeveelheid *M. hyopneumoniae* DNA in de longspoelsels werd gedetecteerd en een significant lagere IL-1ß en IL-6 concentratie werd aangetroffen in de longspoelsels vergeleken met de niet gevaccineerde geïnfecteerde varkens.

Na het uitvoeren van deze studies kunnen we besluiten dat er een hoge *M. hyopneumoniae* diversiteit werd aangetroffen in de loten slachtvarkens van Vlaamse varkensbedrijven en dat een hogere pneumonie en fissuren prevalentie en ergere *Mycoplasma*-like longlaesies worden aangetroffen in loten waar er meer dan één stam wordt aangetroffen. Het respiratoir systeem kan worden aangetast door $PM_{10}$ en $NH_3$, alhoewel het effect van $PM_{10}$ hier in onze studie belangrijker bleek. Onder de omstandigheden van een experimentele studie oefende DON in de concentratie van 1,800 µg/kg geen effect uit op een *M. hyopneumoniae*-infectie. Het commercieel vaccin dat gebruikt werd in het laatste hoofdstuk bleek zeer doeltreffend te zijn tegen een challenge infectie met twee genetisch verschillende en recente *M. hyopneumoniae* isolaten. Niettemin, kon het vaccin niet verhinderen dat het ademhalingsstelsel van de geïnfecteerde varkens gekoloniseerd werd.

Verder onderzoek kan zich richten op factoren die het ontstaan van *M. hyopneumoniae* diversiteit uitlokken en waarom $PM_{10}$ het respiratoir stelsel van het varken meer blijkt te beïnvloeden vergeleken met $NH_3$. Een ander interessant gegeven om verder te onderzoeken is het effect van DON op de gezondheid van het varken en specifiek het effect van DON op een *M. hyopneumoniae* infectie onder veldomstandigheden, aangezien er een mogelijkheid bestaat dat een multi-mycotoxine en multi-pathogene problematiek in het veld deze interacties kunnen beïnvloeden. Verder onderzoek naar nieuwe vaccins die het varken nog een betere bescherming tegen *M. hyopneumoniae*-infecties kunnen bieden is aangewezen, alhoewel dat het opklaren van de pathogenese van *M. hyopneumoniae* wellicht een must is om dit verder te kunnen verbeteren.

## CURRICULUM VITAE

# *Curriculum Vitae*

# CURRICULUM VITAE

Annelies Michiels was born in Bruges on the 12th of March in 1985. She graduated in 2011 as a master in Veterinary Sciences at Ghent University.

Bitten by research she started a position at CODA-CERVA in October 2011. In February 2012 she started the PhD program in a position as researcher on the PIGDUST IWT-project and started the research on *M. hyopneumoniae* in the Unit Porcine Health Management form the Department of Reproduction, Obstetrics and Herd Health in collaboration with the Department of Pathology, bacteriology and poultry diseases. From October 2012 onwards she became a resident of the European college of Porcine Health Management. In October 2013 she started a position as assisting academic staff in the Unit Porcine Health Management. To support her teaching activities as an academic assistant, she successfully finished the specific academic teacher program in 2014 at CVO Panta Rhei. In 2017 she started the Specialist Pig Veterinarian Course at Ghent University.

Annelies is first author and co-author of several studies published in international peer reviewed journals. Her experimental work has been presented in different national and international congresses.

## BIBLIOGRAPHY

# *Bibliography*

# BIBLIOGRAPHY

## Publications in national and international journals

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# DANKWOORD

# *Dankwoord*

# DANKWOORD

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